Contents

Articles in English, Abstracts in English and Czech or Slovak

Volume 75 September 2006 Number 3

ACTA VET. BRNO 2006, 75: 319-328; doi:10.2754/avb200675030319

Biological Responses of Antiprogestins in Mammary Gland, Uterus and Seminal Vesicles of Prepubertal Intact and Adult Gonadectomized Mice

E. KÖHLEROVÁ, J. KOTANOVÁ, J. ŠKARDA

Institute of Animal Physiology and Genetics, Academy of Sciences of the Czech Republic, Prague

Received December 1, 2005
Accepted March 16, 2006

Abstract

Köhlerová E., J. Kotanová, J. Škarda: Biological Responses of Antiprogestins in Mammary Gland, Uterus and Seminal Vesicles of Prepubertal Intact and Adult Gonadectomized Mice. Acta Vet. Brno 2006, 75: 319-328.

The present study tested and compared the antiproliferative and proliferative activities of three antiprogestins on four separate mouse model systems: prepubertal intact and adult ovariectomized (OVX) females, prepubertal intact and adult castrated males. In prepubertal intact males and females and adult castrated males, norethindrone acetate (NA; a synthetic steroid exhibiting progestational and estrogenic activities)- stimulated mammary growth was decreased by antiprogestins: more by RU 46556 (RU) than RU 38486 (MI) and onapristone (ON) . In adult OVX females the inhibitory effect of RU and MI was lower than that of ON. Uterine weights were not significantly decreased by MI, were significantly decreased by RU at a lower daily dose (50 μg) but not affected by a high (500 μg) dose. Seminal vesicle weights were increased by RU but not affected by MI in both NA-treated prepubertal and adult castrated males. In adult castrated but not in prepubertal males ON decreased seminal vesicle weights. In 17β-estradiol (E) plus progesterone (Prog)-treated animals of all four model systems, RU (100 μg/d) acted additively with a submaximal daily dose (10 μg) of antiestrogen ICI 182, 780 (ICI) to produce a lower mammary gland growth rate than ICI alone. In uterus, however, no significant effect of a combination of ICI plus RU was noted when compared with ICI alone. In general, our assay could serve as an *in vivo* tool for the detection of steroid hormone agonist and antagonist activities of newly synthesized analogs of steroid hormones, and natural and man-made chemicals and extracts of environmental samples.

Mifepristone, RU 46556, onapristone, growth, uterus, seminal vesicles, mice, bioassay

Progesterone (Prog) is a key modulator of the cellular processes that are required for the development and maintenance of reproductive function. Produced primarily by ovarian granulosa cells, it mediates its biological activity throughout the body by interacting with specific high- affinity nuclear Prog receptors (PR) proteins located in target cell nuclei. In the absence of ligand, PR resides in the nucleus associated with a high molecular weight complex comprising heat shock proteins (Hsps) and possibly other proteins. Upon binding of Prog, PR undergoes a conformational change and initiates the signal transduction cascade by phosphorylation, promoting displacement of Hsps, dimerization, nuclear translocation, the interaction of the receptor dimer with specific Prog response elements (PREs) located within target gene promoters and recruitment of adaptor proteins, which allow PR to interact productively with the general transcription apparatus (McDonnell 1995). The magnitude of the positive or negative effects of the DNA bound receptor on transcription is subsequently determined by the structure of the PR complex, promoter environment and the expression level of the receptor associated proteins such as coactivators and corepressors. Thus, PR may not function in an equivalent manner in all cells (Sathya et al. 2002). There is now ample evidence that a steroid hormone may interact with various isoforms or variations of nuclear receptors. Thus, Prog may be bound to either PR A, B or C. All forms of PR are derived from a single gene as a consequence of alternate initiation of transcription

Address for correspondence:
Doc. MVDr. Josef Škarda, DrSc.
Institute of Animal Physiology and Genetics,
Academy of Sciences of the Czech Republic
Vídeňská 1083, 142 20 Praha 4, Czech Republic

Phone: +420 267 09 05 05
Fax: +420 267 09 05 00
E-mail: skarda@iapg.cas.cz
http://www.vfu.cz/acta-vet/actavet.htm

from distinct promoters (Kastner et al. 1990; Wei et al. 1996; Giangrande et al. 1997). PR-B is primarily involved in mediating the positive transcriptional responses to Prog, whereas PR-A is, with few exceptions, inactive under the same conditions and moreover in several systems the primary function of PR-A appears to be a negative regulator of PR-B mediated transcription. PR-C can modulate the transcriptional activity of both PR-A and PR-B. Significantly, it was observed also that the transcriptional activity of the glucocorticoid, mineralocorticoid, androgen and estrogen receptors (ER) was negatively regulated by PR-A (Vegeto et al. 1993). The contrasting activities of PRs suggest that the relative tissue distributions of the three receptor isoforms are a major determinant of the pleitropic biological effects of progestins and antiprogestins. Antiprogestins compete with Prog for binding to PR without inducing complete signal transduction. Since inhibition of transcriptional activity by steroid receptor antagonist may vary, some antihormones have a partial agonistic activity. In addition, expression of agonistic and antagonistic activity varies between hormone dependent tissues, depending on the spectrum of locally available nuclear receptors, coactivators and corepressors.An active genomic Prog response mechanism has been found to coexist with the nongenomic one in reproductive tissues (Revelli et al. 1998).

Detection and characterization of agents as steroid hormone agonist and antagonist necessitates the integrated use of *in vivo* and *in vitro* assays. Recently, we have tested and compared four mouse model systems the prepubertal intact female, the adult ovariectomized (OVX), the prepubertal male and adult castrated male for determination of steroid hormone agonist and antagonist activities *in vivo*. It was based on simultaneous evaluation of a combination of several endpoints (mammary, uterine, seminal vesicles and spleen growth responses). The changes in organ growth were determined following administration of a tested compound alone or in combination with hormones known to stimulate or inhibit organ growth (Škarda 2002abc, 2003; Hufriy et al. 2003; Köhlerová and Škarda 2004; Kotanová et al. 2005; Škarda and Köhlerová 2006).

The purpose of the present experiments was to test the ability of four model systems to evaluate the proliferative and antiproliferative activities of three antiprogestins and a combination of antiprogestin and antiestrogen.

Materials and Methods

Materials

Progesterone (Prog), 17b estradiol (E), norethindrone acetate (NA), t-amylalcohol and methylsalicylate were purchased from Sigma-Aldrich (Prague, Czech Republic). 2,2,2-tribromoethanol (Fluka Chemie AG, Buchs, Switzerland) was bought for preparation of Avertin. Hematoxylin was bought from Merck (Darmstadt, Germany). Steroidal antiestrogen Faslodex (ICI 182,780; ICI) was obtained both as a gift from Zeneca Pharmaceuticals, Maclesfield (UK) and as goods purchased from Tocris Cookson Ltd., Northpoint Fourth Way Avonmonth (UK). Antiprogestins RU 38486 (Mifepristone; MI) and RU 46556 (RU) were gifts from the Centre de Recherches Rousell-Uclaf, Romainville (France) and ZK 98.299 (Onapristone; ON) was a gift from Schering AG, Berlin (Germany). Hormones and antihormones were dissolved in oily vehicle (Köhlerová and Škarda 2004). Heavy-duty Kapak/Scotchpak heat sealable pouches (Stock no. 502) were purchased from KAPAK Corporation (Minneapolis, MN, U.S.A).

Animals

Outbred C3H mice were from our own colony. Animals were maintained on a 12 h light/12 h dark lighting schedule (light 6.00-18.00 h), fed pelleted nutritionally complete diet (TOP Velas, Lysá n. Labem, Czech Republic). The diet and water were allowed *ad libitum* and the daily routine in the vivarium was constant. To minimize variation of the dose-response relationship of injected hormones, all young animals were weighed at the age of 18 days and only animals weighing 8.5 ± 1g were randomly divided into the treated groups or used for further breeding.

Young intact (at 18 days of age) and adult gonadectomized (females were ovariectomized at 22-24 days of age, i.e. before the allometric growth phase of the mammary gland, males were castrated at 30-35 days of age) mice were used. Gonadectomy was performed under Avertin anesthesia and administration of compounds began 15-20 days after operation. Animals were injected 50 μl of oily vehicle (control) or a compound in oily vehicle s.c. for 10 or 15 days in females and males, respectively. Doses of hormones are expressed in μg per day *pro toto*. The mean

weight of young intact females was 11g, young intact males 13 g, adult OVX females 21 g, adult castrated males 24 g. The period of hormone treatment is long enough to stimulate/inhibit mammary gland and uterine growth and mimic exposure to a constant level of a compound for a significant phase of life much like that achieved in animals exposed to hormonally active xenobiotics. At the end of treatment and within 24 h of the last hormone administration, animals were weighed and killed. The first pair of inguinal mammary fat pads was removed for whole-mount. Mammary fat pads of males that showed no detectable mammary gland (10%) were excluded from the data set; not detectable mammary gland was usually from one side, mammary gland from the other side was used for evaluation. Uteri and seminal vesicles were dissected out and weighed wet on a precision electronic balance. Weights of uteri and seminal vesicles before expressing the fluid secretion are presented. All experimental procedures were conducted in compliance with the highest standards of humane animal care and approved by the Ethical Committee of the Institute of Animal Physiology and Genetics of the Academy of Sciences.

Mammary whole-mount preparation and quantitative mammary histology

Mammary fat pads spread as flat as possible, put into Carnoy's fixative, defatted in acetone, stained in hematoxylin, destained in 1% HCl, blued in 0.1% NH_4OH, dehydrated through graded alcohols, cleared in toluene and stored in methyl salicylate in a heavy duty heat sealed pouches (Köhlerová and Škarda, 2004).

A modification of Chalkley's morphological analysis was used to determine the percentage area of the mammary fat pad occupied by mammary epithelial structures on enlarged (×12) photographs of the mammary whole-mount preparation (Škarda 2003).

Statistical analysis

All data represent mean ± SEM. Statistically significant difference was determined by ANOVA, followed by the Bonferroni test for individual comparisons of the mean values.

Results

Effects of RU, MI and ON in females

In young intact females RU caused a significant 51%, 47%, and 69% inhibitions of NA (12.5 μg/d) - stimulated mammary growth at 50, 100 and 500 μg doses, respectively. MI, on the other hand, at the same doses caused only 36%, 34% and 38% decreases in mammary growth (Fig. 1).

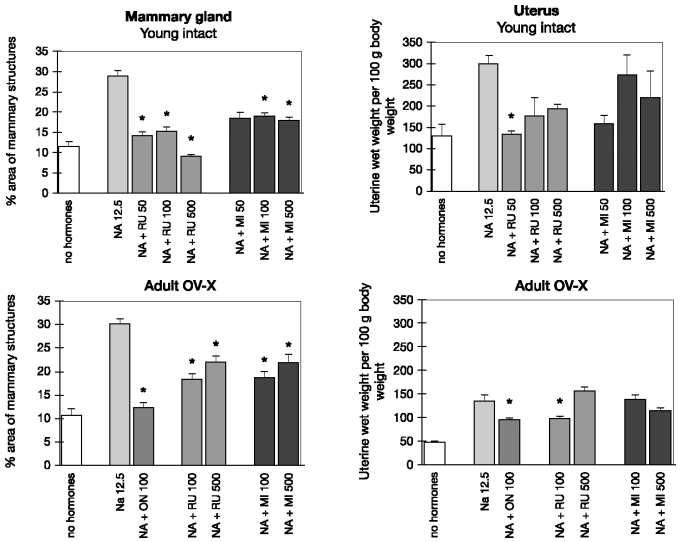

Fig. 1. Effects of progesterone antagonists RU 46556 (RU), mifepristone (MI) and onapristone (ON) on norethindrone acetate (NA) stimulated mammary and uterine growth in female mice. Prepubertal (day 18 of age) and adult OVX (ovariectomized at 22-24 days of age) females (4-5 per group) received s.c. injections of vehicle (no hormones) or different daily doses of hormones (given in μg/d) for 10 days. Results represent the mean ± SEM.
* $p < 0.05$

RU at the lowest daily dose (50 μg) decreased uterine weight significantly by 55%, whereas doses of 100 and 500 μg non- significantly. MI at all used doses decreased uterine weight non-significantly, obviously due to high variability in uterine weights.

In adult OVX females RU, MI and ON caused significant inhibitions of NA-stimulated mammary growth. ON and RU at daily dose 100 μg caused significant decreases in uterine weights, whereas MI had no effect.

Effects of RU, MI and ON in males

In prepubertal intact males, ON had no significant effect at the lowest daily dose (50 μg) used and caused 35% and 83% decreases in NA (12.5 μg/d) stimulated mammary growth at doses of 100 and 500 μg/d, respectively. RU caused 83%, 85%, and 93% and similarly MI caused a 79%, 86% and 84% inhibitions of NA stimulated mammary growth at doses of 50, 100, and 500 μg/d, respectively (Table 1). When mammary growth was stimulated by NA at a daily dose of 25 μg, RU caused 90%, 89%, and 91% inhibitions, whereas MI caused 87%, 82%, and 85% inhibitions of NA stimulated mammary growth.

Table 1. Effect of progesterone antagonists ON, RU and MI on NA- stimulated mammary growth and seminal vesicle and spleen weights in prepubertal intact C3H male mice

Hormonal treatment ($\mu g.d^{-1}$)	The % area of mammary fat pad occupied by mammary epithelial structures	Seminal vesicle weights (mg.100 g^{-1} of body weight)	Spleen weight (mg.100 g^{-1} of body weight)
0	1.0±0.1 (30)	130.6±12.1 (15)	557.4±20.5 (15)
NA 12.5	17.5 ± 1.4^{A} (20)	143.5 ± 11.0^{A} (8)	575.7 ± 34.3^{A} (9)
NA + ON 50	13.3 ± 2.9^{A} (8)	86.9 ± 9.8^{A} (5)	577.8 ± 18.9^{A} (5)
NA + ON 100	11.3 ± 1.0^{B} (10)	76.6 ± 4.7^{A}(5)	562.3 ± 31.3^{A} (5)
NA + ON 500	2.9 ± 0.4^{B} (10)	78.9 ± 7.4^{A}(5)	669.0 ± 35.3^{A} (5)
NA 12.5	17.5 ± 1.4^{A} (20)	143.5 ± 11.0^{A} (8)	575.7 ± 34.3^{A} (9)
NA + RU 50	2.9 ± 0.3^{B} (14)	153.8 ± 21.6^{A} (8)	623.2 ± 51.5^{A} (8)
NA + RU 100	2.7 ± 0.3^{B} (22)	159.3 ± 16.6^{A} (11)	537.2 ± 29.6^{A} (11)
NA + RU 500	1.3 ± 0.2^{B} (20)	234.2 ± 17.8^{B} (10)	470.1 ± 15.6^{A} (10)
NA 12.5	17.5 ± 1.4^{A} (20)	143.5 ± 11.0^{A} (8)	575.7 ± 34.3^{A} (9)
NA + MI 50	3.7 ± 0.5^{B} (9)	170.9 ± 13.1^{A} (5)	570.1 ± 42.0^{A} (5)
NA + MI 100	2.5 ± 0.6^{B} (11)	217.9 ± 12.6^{A} (5)	481.3 ± 15.9^{A} (5)
NA + MI 500	2.8 ± 0.9^{B} (10)	128.0 ± 18.9^{A} (5)	552.5 ± 46.0^{A} (5)
NA 25	32.9 ± 2.3^{A} (10)	71.8 ± 15.9^{A} (5)	672.1 ± 39.0^{A} (5)
NA + RU 50	3.3 ± 0.4^{B} (11)	142.6 ± 12.0^{A} (6)	544.9 ± 45.1^{A} (5)
NA + RU 100	3.6 ± 0.5^{B} (12)	209.6 ± 12.9^{B} (5)	560.0 ± 18.6^{A} (6)
NA + RU 500	2.9 ± 0.7^{B} (10)	283.7 ± 20.7^{B} (5)	499.2 ± 10.1^{B} (5)
NA 25	32.9 ± 2.3^{A} (10)	71.8 ± 15.9^{A} (5)	672.1 ± 39.0^{A} (5)
NA + MI 50	4.3 ± 0.7^{B} (8)	86.0 ± 16.3^{A} (4)	524.6 ± 20.5^{A} (4)
NA + MI 100	5.9 ± 1.1^{B} (8)	86.9 ± 31.6^{A} (4)	666.9 ± 41.4^{A} (4)
NA + MI 500	5.1 ± 1.1^{B} (7)	85.8 ± 10.7^{A} (4)	453.2 ± 50.7^{B} (4)

Prepubertal intact (day 18 of age) males were injected s.c. with of 50 μl vehicle (0; control) or NA or NA plus anti-progestins for 15 days. Values are given as means ± SEM. Values within a columm, which do not have the same upper case superscript (A, B) differ significantly ($p < 0.05$). Numbers in parentheses indicate the number of the first inguinal mammary glands, or number of animals supplying seminal vesicles and spleen. Other details are given in the legend to Fig.1.

In prepubertal intact males, ON and MI had no significant effect on seminal vesicle weights in NA-treated animals. RU (500 μg/d), on the other hand, caused 63% increase in

seminal vesicle weight in animals treated with NA at a daily dose of 12.5 μg and caused 192% and 295% increase at doses of 100 and 500 μg/d, respectively, in animals treated with Na at a daily dose of 25μg.

Spleen weights of prepubertal intact males treated with NA at a daily dose of 12.5 μg were not affected by simultaneous treatment with ON, RU and MI, whereas in animals treated with NA at a daily dose of 25 μg, spleen weights were decreased by the highest daily dose (500 μg) of both RU (by 26%) and MI (by 33%).

In adult castrated males NA-stimulated (12.5 μg/d) mammary growth was inhibited by ON at a daily dose of 500 μg by 76%. RU caused 67%, 69%, and 81% inhibitions of mammary growth at doses of 50, 100, and 500 μg/d, respectively. Mammary growth stimulated by a high dose of NA (50 μg/d) was decreased by RU and MI at daily doses of 50 - 500 μg by 86 - 94% (Table 2).

Table 2. Effect of progesterone antagonists ON, RU and MI on NA-stimulated mammary gland and seminal vesicles growth in adult castrated C3H male mice

Hormonal treatment ($\mu g.d^{-1}$)	The % area of mammary fat pad occupied by mammary epithelial structures	Seminal vesicle weights (mg.100 g^{-1} of body weight)	Spleen weight (mg.100 g^{-1} of body weight)
0	1.3±0.3 (24)	37.5±3.9 (12)	403.2±7.6 (12)
NA 12.5	7.2 ± 1.2^{A} (24)	44.3 ± 2.9^{A} (12)	395.9 ± 31.6^{A} (12)
NA + ON 500	1.7 ± 0.5^{B} (6)	21.5 ± 1.7^{B} (3)	498.6 ± 46.6^{A} (3)
NA 12.5	7.2 ± 1.2^{A} (24)	44.3 ± 2.9^{A} (12)	395.9 ± 31.6^{A} (12)
NA + RU 50	2.4 ± 0.4^{B} (12)	47.0 ± 6.5^{A} (6)	410.1 ± 27.9^{A} (6)
NA + RU 100	2.2 ± 0.3^{B} (10)	83.1 ± 8.5^{B} (5)	397.0 ± 36.1^{A} (5)
NA + RU 500	1.4 ± 0.4^{B} (4)	$209.4\pm34.4^{*}$ (2)	$456.3\pm35.5^{*}$ (2)
NA 50	37.6 ± 2.8^{A} (16)	78.7 ± 4.3^{A} (8)	456.8 ± 36.9^{A} (8)
NA + RU 50	3.7 ± 0.8^{B} (8)	109.7 ± 10.2^{A} (4)	501.2 ± 56.1^{A} (4)
NA + RU 100	3.2 ± 0.8^{B} (8)	135.5 ± 15.1^{B} (4)	497.2 ± 20.3^{A} (4)
NA + RU 500	$2.4.\pm0.7^{B}$ (8)	239.0 ± 17.1^{B} (4)	715.9 ± 161.1^{A} (4)
NA 50	37.6 ± 2.8^{A} (16)	78.7 ± 4.3^{A} (8)	456.8 ± 36.9^{A} (8)
NA + MI 50	5.2 ± 0.9^{B} (10)	80.6 ± 7.3^{A} (5)	384.1 ± 35.5^{A} (5)
NA + MI 100	3.1 ± 0.7^{B} (8)	58.1 ± 4.6^{A} (5)	453.8 ± 35.5^{A} (5)
NA + MI 500	2.7 ± 0.4^{B} (10)	70.6 ± 3.1^{A} (5)	430.9 ± 25.4^{A} (5)

Animals were castrated at 30-35 days of age and administration of compounds begun 15-20 days after operation.
* two animals from the group were excluded from the data set due to difficulties with seminal vesicle and spleen dissection resulting from the post-castration inflammatory reaction (peritonitis)
Other details are given in Table 1.

Seminal vesicle weights were decreased by high doses of ON, increased by RU and not affected by MI. In animals treated with NA (12.5 μg/d), the daily 500 μg dose of ON caused a 43% decrease in seminal vesicle weights, whereas RU exerted significant stimulatory effect of this variable and led to 88% and 393% increases at doses of 100 and 500 μg/d, respectively (two animals from the group treated with 500 μg/d of RU were excluded from the data set due to difficulties with seminal vesicle dissection resulting from the post-castration inflammatory reaction). Similarly, in animals treated with a high dose of NA (50 μg/d), RU at daily dose 100 and 500 μg caused 72% and 204% increases in seminal vesicle weights, respectively.

Spleen weights of adult castrated males were not affected by treatment with antiprogestins.

Table 3. Effects of antiestrogen ICI 182,720 (ICI) alone and in combination with antiprogestins RU on mammary growth and uterine, seminal vesicle and spleen weights in E plus Prog treated female and male mice. Other details are given in the Fig.1 and Table 1.

Hormonal treatment ($\mu g.d^{-1}$)	The % area of mammary fat pad occupied by mammary epithelial structures		Uterus/seminal vesicle weights (mg.100 g^{-1} of body weight)		Spleen weight (mg.100 g^{-1} of body weight)	
FEMALE	Intact	OV-X	Intact	OV-X	Intact	OV-X
0	13.1±0.8 (16)	11.0±1.5 (12)	107.1±6.7 (7)	29.6±1.7 (6)	612.5±19.9 (7)	562.3±16.2 (6)
E 0.01+Prog 500	28.7±0.9^{A} (10)	41.0±2.1^{A} (12)	269.7±16.1^{A} (5)	159.1±12.2^{A} (6)	699.7±15.7^{A} (7)	554.4±15.4^{A} (6)
E+Prog+ICI 10	18.5±1.6^{BCE} (12)	24.2±1.8^{BCE} (12)	99.2±3.0^{BCE} (6)	71.9±5.2^{BCE} (6)	750.1±25.2^{A} (6)	508.7±22.3^{A} (6)
E+Prog+ICI+RU100	4.5±0.7^{BD} (12)	11.6±0.6^{BD} (10)	126.6±7.2^{BC} (6)	80.0±5.0^{BC} (6)	667.8±32.7^{A} (6)	524.1±23.2^{A} (6)
E+Prog+ICI 50	3.9±0.5^{BF} (10)	13.3±1.1^{BF} (12)	87.9±5.7^{BE} (5)	40.8±3.6^{BF} (6)	693.7±31.3^{A} (6)	533.5±20.8^{A} (6)
MALE	Intact	Castrated	Intact	Castrated	Intact	Castrated
0	1.4±0.6 (12)	0.7±0.1 (12)	196.0±14.2 (6)	33.3±4.4 (6)	559.0±22.2 (6)	457.9±18.1 (6)
E 0.05+Prog 500	37.3±1.1^{A} (12)	39.3±4.2^{A} (10)	43.3±4.1^{A} (5)	17.5±3.1^{A} (6)	679.1±34.0^{A} (5)	480.9±26.2^{A} (6)
E+Prog+ICI 10	13.8±1.8^{BCE} (11)	18.4±2.3^{BCE} (12)	110.8±18.6^{ADE} (4)	14.6±1.5^{ADE} (6)	636.0±33.4^{A} (6)	458.4±18.1^{A} (6)
E+Prog+ICI+RU100	3.6±0.8^{BD} (9)	2.3±0.6^{BD} (8)	285.1±32.4^{BD} (6)	57.6±2.8^{BD} (6)	541.1±43.5^{A} (6)	402.7±10.3^{A} (5)
E+Prog+ICI 50	3.1±0.6^{BF} (9)	1.3±0.2^{BF} (9)	240.0±29.7^{BF} (6)	17.9±1.6^{AE} (6)	618.8±13.8^{A} (6)	409.3±6.5^{A} (6)

Effects of a combination of RU plus ICI

E (0.01 μg/d) plus Prog (500 μg/d)-stimulated mammary growth was decreased by a sub-maximal daily dose of ICI (10 μg) by 36% and 41% in prepubertal intact and adult OVX females, respectively. A combination of ICI and RU (100 μg/d) caused 84% and 72% decreases in E plus Prog-stimulated mammary growth in prepubertal intact and adult OVX females, respectively. However, the same level of inhibition of mammary growth was obtained by a high dose (50 μg/d) of ICI alone.

E plus Prog-stimulated uterine weight was significantly decreased by ICI at daily doses of 10 μg and 50 μg by 63% and 67% in prepubertal intact and by 55% and 74% in adult OVX females, respectively. A combination of ICI and RU had no effect on uterine growth in both prepubertal intact and adult OVX females (Table 3).

E (0.05 μg/d) plus Prog (500 μg/d)-stimulated mammary growth was decreased by ICI (10 μg/d) by 63% and 53% in prepubertal intact and adult castrated males, respectively. A combination of ICI and RU caused 90% and 94% decreases in E plus Prog-stimulated mammary growth in young intact and adult castrated males, respectively. ICI alone at a high daily dose (50 μg) had approximately the same effect on E plus Prog-stimulated mammary growth as the above mentioned combination of ICI plus RU.

ICI (10 μg/d) caused 156% increase in seminal vesicle weights of E plus Prog-treated prepubertal intact males. RU (100 μg/d) acted synergistically with ICI to produce higher seminal vesicle weight

(588% increase) than that produced with a moderate dose of ICI alone. However, the high dose of ICI (50 μg/d) alone increased seminal vesicle weight by 454% that is not significantly different from that obtained by a combination of ICI plus RU. No such stimulatory effect of ICI was noted in seminal vesicles of castrated males.

Discussion

NA is a synthetic steroid exhibiting mainly progestational and estrogenic activities. In moderate doses (12.5-25.0 μg/d in females and 50 μg/d in males) it acts in mice as a combination of a low dose of E and a high dose of Prog (Škarda 2002c), and thus, it was used to demonstrate the abilities of our *in vivo* model to detect not only antiprogestational and antiestrogenic activities of antiestrogens and antiprogestins, but also their other possible hormone agonistic, antagonistic or synergistic capabilities.

Unlike the available ER-antagonists that are highly receptor selective, the available PR-antagonists also interact with the receptors for glucocorticoids, mineralocorticoids, androgens and estrogens (Philibert et al. 1989; Chwalisz et al. 1998; Sathya et al. 2002). The pure antiprogestational agent has not yet been synthetized. Two types of antagonists of the signal transduction pathway of Prog were developed and are currently being used for the treatment of a variety of endocrine-related disorders. Formally it was believed that Type I compounds failed to promote binding of PR to PREs whereas Type II antagonists did bind. Currently it is believed that Type I antagonist (e.g.ON) binds to PREs but promotes a conformational change distinct from that induced by Type II antagonists (e.g. MI or RU). The pharmacology of ON indicates that it is a more complete antagonist than MI. The cross-talk with cAMP signalling pathways potentiates the agonist activity of MI but has no effect on the biological activity of ON (Gass et al. 1998; Spitz 2003). The human PR- MI complex recognizes PREs and blocks the hormone-dependent transcriptional activation function of TAF-2 but not that of hormone-independent TAF-1, permitting the cell and promoter specific expression of partial agonist activity (Meyer et al. 1990).

MI (13β- methyl-substituted antiprogestin) is known to possess strong antiprogesterone and antiglucocorticoid along with moderate antiandrogen properties. In search for more dissociated derivates, the hydroxy substituent and the propynyl group in position 17 of the MI was replaced by a spiroether group, which is known to induce specific affinity for the PR. Dimethyl amino group on the 11β-phenyl was replaced by methylthio group. The new derivative RU was about three times more active than that of MI for inducing abortion in rats and was devoid of any antiglucocorticoid activity on the thymus weight in rats. However, in contrast to MI, it has significant androgenic activity on rat prostate and also exhibits a slight progestomimetic activity on rat endometrial proliferation (Philibert et al. 1989, 1991; Chwalisz et al. 1998). ON is the 13α-methyl-substituted antiprogestin that shares a number of structural similarities to MI; ON being less antiglucocorticoid than MI. In the rat uterus, it rapidly upregulates several genes normally under estrogenic regulation (Chwalisz et al. 1998). Prog is a major sex steroid controlling estrogen action in reproductive tracts and other estrogen-dependent tissues, therefore, it is not surprising that antiprogestins also interfere with various estrogenic responses. The antiproliferative effects of antiprogestins were not seen to the same degree in the uterus of E-treated OVX females of different species. MI and ON can block the ability of E to increase endometrial growth in intact and OVX non-human primates and rabbits (Chwalisz et al. 1991; Burleigh et al. 1998). However, in E-treated OVX rats, ON and MI did not block E-stimulated endometrial growth, and luminal and glandular epithelium were stimulated more after antiprogestin plus E, than after E alone. ON but not MI reduced E-stimulated myometrial proliferation and induced an overall uterine weight reduction (Chwalisz et al. 1998). Burleigh et al.

(1998) showed that MI altered uterine weight only in rats receiving pharmacological doses of E. In NA-treated (25 μg/d) prepubertal (present results) and adult OVX female mice, uterine weights were not significantly affected by MI, however they were decreased by RU and ON at lower daily doses (50 μg in prepubertal females; 100 μg in adult OVX females) and not affected by high (500 μg/d) dose. Based on these results, use of mouse uterine weight bioassay for screening and identification of antiprogestins is not recommended but it is still suitable for characterization of newly synthetized antiprogestins. Unlike MI, which in our experiments had no antiandrogenic activities measured according to seminal vesicle weights, RU displayed a full androgenic effect at a daily dose of 100 μg and 500 μg in both prepubertal and adult castrated male mice similarly as in the rat prostate (Philibert et al. 1989, 1991). The fact that the same molecule of antiprogestin may also exhibit antiglucocorticoid (MI), androgenic (RU; RU 49295), antiandrogenic (MI) or progestomimetic (RU 49295) effects (Philibert et al. 1989, 1991) may explain variable responses of uterus and seminal vesicles to ON, MI and RU in our experiments. In the present experiments, the mammary gland was shown to be the most suitable tissue for the measurement of the antiproliferative potency of Prog antagonists *in vivo*. The bioassay depends on the inhibition of the well known stimulating effect of Prog on mammary duct lateral branching and on the formation of tubulo-alveolar buds, which are determined by highly sensitive morphometric analysis. RU was more active than MI as antiprogestin in the mammary gland of NA-treated prepubertal female and male mice. ON was not consistently more or less active than RU and MI. Similar results were obtained by Michna et al. (1991) in OVX rats.

However, for the measurement of the antiproliferative potency of antiprogestins it is essential to substitute the animals with Prog and estrogen (or NA), since the stimulating effect of Prog on the mammary gland is only induced when both hormones are given. The fact that the growth-stimulating potency of Prog necessitates the priming effect of estrogens suggests that antiestrogens should be able to decrease growth stimulating potency of Prog by blocking the priming effect of estrogen. Indeed, in our mouse models, E plus Prog-or NA-stimulated growth of mammary epithelial structures was inhibited by both antiprogestins and antiestrogens. In fact, antiprogestins significantly inhibited the growth of mammary ducts in mice treated with E only (our unpublished results), although these compounds are known to have low or no affinity to the ER (Henderson 1987). These facts show that *in vivo* bioassay of antiprogestins is not fully specific but it may be verified by *in vitro* antiprogestin bioassay developed by Taylor et al. (1996). These authors developed a rapid method of screening anti-progestational agents in the primary culture system of virgin rat mammary epithelial cells grown in a serum-free medium on gels of rat tail collagen. In this culture conditions epithelial cells respond with a decrease in DNA synthesis that is dose-dependent and reproducible over a wide dose range.

In the mammary gland of E plus Prog treated animals, RU acted additively with a submaximal dose of ICI to produce a lower growth rate than that observed in animals treated with ICI alone. However, uterine weights were decreased by ICI and not affected by further addition of RU. Such antiproliferative properties have importance with regard to treatment with antiestrogens and antiprogestins and implications for their potentional use for prevention and treatment of mammary and uterine cancers in human and veterinary medicine.

In conclusion, the measurement of antiproliferative potency of antiprogestins on the mammary gland of male and female mice treated with E plus Prog or NA is recommended for *in vivo* screening of antiprogestins. The other endpoints - uterine, seminal vesicle and spleen weights give only supplementary information on the other hormone agonist or antagonist activities of tested compounds.

Biologické účinky antiprogestinů v mléčné žláze, uteru a semenných váčcích prepubertálních a dospělých gonadektomovaných myší

Předložená práce testuje a srovnává antiproliferativní a proliferativní účinky tří antiprogestinů na čtyřech myších modelech: prepubertální intaktní a dospělé ovariektomované (OVX) samice, prepubertální intaktní a dospělí kastrovaní samci. Norethindronem acetátem (NA, syntetický steroid vykazující progestagenní a estrogenní aktivity)-stimulovaný růst mléčné žlázy intaktních prepubertálních samic a samců byl snížen a to více antiprogestinem RU 46556 (RU) než antiprogestinem RU 38486 (MI) a onapristonem (ON). U dospělých OVX samic měl RU a MI nižší inhibiční účinek na NA-stimulovaný růst mléčné žlázy než ON. Hmotnost dělohy nebyla ovlivněna MI, ale signifikantně snížena RU v dávce 50 μg/d u prepubertálních a v dávce 100 μg/d u dospělých OVX samic. Hmotnost semenných váčků nebyla ovlivněna MI, ale zvýšena RU jak u prepubertálních tak u dospělých kastrovaných samců. ON hmotnost semenných váčků snížil signifikantně u dospělých kastrovaných, nesignifikantně u prepubertálních samců. Estradiolem 17β (E) plus progesteronem (Prog) stimulovaný růst mléčné žlázy byl snížen kombinací antiprogestinu RU (100 mg/d) s antiestrogenem ICI 182, 780 (ICI; 10 μg/d) více než samotným ICI. Tento účinek lze charakterizovat jako aditivní, poněvadž vysokou dávkou samotného ICI (50 μg/d) bylo dosaženo stejného efektu jako kombinací RU plus ICI. Hmotnost dělohy jak prepubertálních tak dospělých OVX samic nebyla kombinací RU a ICI ovlivněna více než samotným ICI. Naproti tomu hmotnost semenných váčků byla aplikací ICI u prepubertálních samců a nikoliv u dospělých kastrovaných samců zvýšena. Předložené biostanovení je vhodným prostředkem pro detekci aktivit agonistů a antagonistů nově syntetizovaných analogů steroidních hormonů, přirozených a syntetických chemikálií a vzorků z vnějšího prostředí.

Acknowledgements

We are grateful to Eva Urbanová for excellent technical assistance, Professor F. Neumann (Schering AG, Berlin, Germany) for onapristone and Professor J.-P. Raymond (Centre de Recherches Roussel Uclaf, Romainville, France) for the gift of RU 38486 and RU 46556. This work was funded by the Grant Agency of the Academy of Sciences of the Czech Republic (Grant No. IBS 5045302 and 1QS 500450557) and Institutional Research Plan IAPG (No. AV0Z 50450515).

References

BURLEIGH DW, WILLIAMS RF, GORDON K, HSIU JG, HODGEN GD 1998: Screening for antiproliferative actions of mifepristone- differential endometrial responses of primate versus rats. Contraception **58**: 45-50

CHWALISZ K, HEGELE-HARTUNG C, FRITZEMEIER KH, BEIER HM, ELGER W 1991: Inhibition of the estradiol-mediated endometrial gland formation by the antigestagen onapristone in rabbits: relationship to uterine estrogen receptors. Endocrinology **129** : 312-322

CHWALISZ K, STOCKEMANN K, FRITZEMEIER KH, FUHRMANN U 1998: Modulation of oestrogenic effects by progesterone antagonists in the rat uterus. Human Reprod Update **4**: 570-583

GASS EK, LEONHARDT SA, NORDEEN SK, EDWARDS DP 1998: The antagonists RU486 and ZK98299 stimulate progesterone receptor binding to deoxyribonucleic acid in vitro and in vivo, but have distinct effects on receptor conformation. Endocrinology **139**: 1905-1919

GIANGRANDE PH, POLLIO G, MCDONNELL DP 1997: Mapping and characterization of the functional domains responsible for the different activity of the A and B isoforms of the human progesterone receptor. J Biol Chem **272**: 32889-32900

HENDERSON D 1987: Antiprogestational and antiglucocorticoid activities of some novel 11β-aryl substituted steroids. In: Pharmacology and Clinical Uses of Inhibitors of Hormone Secretion and Action (Furr BJA, Wakeling AE eds.), London, Bailliere Tindall, pp. 184-211

HUFRIY A, KÖHLEROVÁ E AND ŠKARDA J 2003: Numbers of terminal end buds and ductal branchings as morphometric criteria for bioassay of activities of steroid hormone agonists and antagonists on the mammary gland. Acta Vet Brno **72**: 483-491

KASTNER P, KRUST A, TURCOTTE B, STROPP U, TORA L, GRONEMEYER H, CHAMBON P 1990: Two distinct estrogen-regulated promoters generate transcripts encoding two functionally different human progesterone receptor forms A and B. EMBO J **9**: 1603-1614

KÖHLEROVÁ E, ŠKARDA J 2004: Mouse bioassay to assess oestrogenic and antioestrogenic compounds: hydroxytamoxifen, diethylstilbestrol and genistein. J Vet Med A **51**: 209-217

KOTANOVÁ J, KÖHLEROVÁ E, ŠKARDA J 2005: Contrasting ability of steroidal (ICI 182780) and non-steroidal (EM-800) antiestrogens to inhibit reproductive organ growth in male and female mice. Acta Vet Brno **74**: 533-541

MCDONNELL DP 1995: Unraveling the human progesterone receptor signal transduction pathway. Insights into antiprogestin action. Trends Endocrinol Metab **6**: 133-138

MEYER M-E, PORNON A, JI J, BOCQUEL M-T, CHAMBON P, GRONEMEYER H 1990: Agonistic and antagonistic activities of RU 486 on the functions of the human progesterone receptor. EMBO J **9**: 3923-3932

MICHNA H, NISHINO Y, SCHNEIDER MR, LOUTON T AND EL ETREBY MF 1991: A bioassay for the evaluation of antiproliferative potencies of progesterone antagonists. J. Steroid. Biochem Molec Biol **38**: 359-365

PHILIBERT D, COSTEROUSSE G, GAILLARD-MOQUILEVSKY M, NEDELEC L, NIQUE F, TOURNEMINE C, TEUTSCH G 1991: From RU 38486 towards dissociated antiglucocorticoid and antiprogesterone. In: Antihormones in Health and Disease. Front.Horm.Res., Karger Basel **19**: 1-17

PHILIBERT D, HARDY M, GAILLARD-MOGUILEWSKY M, NIQUE F, TOURNEMINE C, NÉDÉLEC L 1989: New analogues of mifepristone with more dissociated antiprogesterone activities. J Steroid Biochem **34**: 413-417

REVELLI A, MASSOBRIO M, TESARIK J 1998: Nongenomic actions of steroid hormones in reproductive tissues. Endocrine Rev. **19**: 3-17

SATHYA G, JANSEN MS, NAGEL SC, COOK CE, MCDONNELL DP 2002: Identification and characterization of novel estrogen receptor-a-sparing antiprogestins. Endocrinology **143**: 3071- 3082

SPITZ IM 2003: Progesterone antagonists and progesterone receptor modulators: an overview. Steroids **68**: 981-993

ŠKARDA J 2002a: Sensitivity and specificity of the bioassay of estrogenicity in mammary gland and seminal vesicles of male mice. Physiol Res **51**: 267-276

ŠKARDA J 2002b: Sensitivity and specificity of bioassay of estrogenicity on mammary gland and uterus of female mice. Physiol Res **51**: 407-412

ŠKARDA J 2002c: Norethindrone acetate and testosterone interactions in mammary gland, uterus and seminal vesicles of mice. Acta Vet Brno **71**: 463-471

ŠKARDA J 2003: Bioassay of steroid hormone agonist and antagonist activities of anti-androgens on mammary gland, seminal vesicles and spleen of male mice. J Vet Med A **50**: 204- 212

ŠKARDA J, KÖHLEROVÁ E 2006: Mouse bioassay for in vivo screening of oestrogen and progesterone antagonists. J Vet Med A **53**: 145-153

TAYLOR JA, FORSYTH IA, WANG M-W 1996: Activity of progesterone and anti-progestins in a rat mammary primary cell culture system. J Steroid Biochem Molec Biol **58**: 117-121

VEGETO E, SHAHBAZ MM, WEN DX, GOOLDMAN ME, O'MALLEY BW, MCDONNELL DP 1993: Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. Mol Endocrinol **7**: 1244-1255

WEI LL, HAWKINS P, BAKER C, NORRIS B, SHERIDAN PL, QUINN PG 1996: An amino-terminal truncated progesterone receptor isoform, PRc, enhances progestin-induced transcriptional activity. Mol Endocrinol **10**: 1379-1387

Adenosine 5'-Monophosphate Aerosol Challenge Does Not Provoke Airflow Limitation in Healthy Cats

K. VONDRÁKOVÁ[1], R. VAN DEN HOVEN[2], R. A. HIRT[2]

[1]Clinic for Dogs and Cats, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic
[2]Clin. Dept. for Small Animals and Horses, Clinic of Internal Medicine and Infectious Diseases, Veterinary University Vienna, Austria

Received August 31, 2005
Accepted May 11, 2006

Abstract

Vondráková K., R. Van Den Hoven, R. A. Hirt: Adenosine 5'-Monophosphate Aerosol Challenge Does Not Provoke Airflow Limitation in Healthy Cats. Acta Vet. Brno 2006, 75: 329-336.

The purpose of our study was to investigate the effects of nebulized adenosine 5'-monophosphate on airflow limitation in healthy cats determined by barometric whole body plethysmography (BWBP), in comparison to the effects of carbachol. Ten healthy 4- to 6-year-old domestic shorthair cats were included in the study. Each cat was placed in a BWBP plexiglass chamber (volume 38 l). Changes in box pressure were measured at baseline and after nebulization of vehicle and increasing concentrations of carbachol and adenosine 5'- monophosphate. Airway responsiveness was monitored as increases in enhanced pause (PENH), a unitless variable derived from dose-response curves estimating airflow limitation. The chosen endpoint was the agonist concentration which increased PENH to 300% of the value obtained after saline nebulization (PCPENH 300). Inter-day repeatability of measurements was assessed by repeated bronchoprovocations with both agonists 2-3 days apart. For carbachol, PCPENH300 was reached in all cats and correlated significantly between days (mean ± SD; 0.54 ± 0.42 mg/ml and 0.64 ± 0.45 mg/ml respectively; $r = 0.58$, $p < 0.05$) In contrast, we found no reaction to adenosine 5'-monophosphate even with the highest concentration nebulized during both measurements. At baseline, mean ± SD PENH was 0.47 ± 0.18 and 0.58 ± 0.24 (measurements 1 and 2), whereas PENH after 500 mg/ml adenosine 5'- monophosphate was 0.46 ± 0.20 and 0.71 ± 0.37. All bronchoprovocation tests were well tolerated by the cats. We conclude that healthy airways in cats do not demonstrate airway responsiveness to inhaled adenosine 5'- monophosphate. This is in agreement with observations in humans as well as our previous findings in dogs, where adenosine 5'- monophosphate had no effect on healthy canine airways, but caused significant airflow limitation after induction of acute bronchitis. To define the value of bronchoprovocation testing with adenosine 5'- monophosphate in the feline respiratory tract, further investigation of this agonist in cats with spontaneous lower airway disease will be required.

Aerosol challenge, adenosine 5'- monophosphate, healthy cats

Inhalational aerosol challenge with measurement of airway responsiveness is considered a valuable diagnostic tool for the detection of lower airway disease. Airway hyperresponsiveness is defined as an abnormal increase in airflow limitation following the exposure to a stimulus and is described as an important pathophysiological characteristic of lower airway diseases (Van Schoor et al. 2000). In addition to humans, this phenomenon was observed in several animal species including mice, rats, guinea pigs, cats and dogs (Chand et al. 1993; Hamelmann et al. 1997; Theodorou et al. 1997; Hofmann et al. 1999; Hannon et al. 2001).

A bronchoprovocative agonist commonly used for aerosol challenge in cats is carbachol, which causes airflow limitation by a direct action on the effector cells involved in the reduction of flow, such as airway smooth muscle cells, bronchial vascular endothelial cells and mucus producing cells. In humans, indirectly acting agonists, such as adenosine

Address for correspondence:
MVDr. Kateřina Vondráková, PhD.
Clinic for Dogs and Cats, Faculty of Veterinary Medicine
University of Veterinary and Pharmaceutical Sciences Brno
Czech Republic

E-mail: vondrakovak@hotmail.com
http://www.vfu.cz/acta-vet/actavet.htm

5'- monophosphate (AMP), are thought to be more specific, since they only cause airflow limitation in inflamed airways. Their mechanism of action is based on cells other than the effector cells; these cells subsequently interact with the aforementioned effector cells. Cells that act as an intermediary between the indirect stimuli and the effector cells are inflammatory cells (e.g. mast cells) and neuronal cells (Van Schoor et al. 2000; Currie et al. 2003). Since there is a lack of effector cells for indirect stimuli in the airways of healthy subjects, no or very weak responses are expected after their administration. In contrast, directly acting agents should cause bronchoconstriction even in subjects without evidence of respiratory inflammation (Polosa and Holgate 1997; Van Den Berge et al. 2001).

Barometric whole body plethysmography (BWBP) is an extremely non-invasive method used for monitoring airway responses to induced bronchoconstriction in rodents, cats or dogs (Chand et al. 1993; Hamelmann et al. 1997; Hofmann et al. 1999; Talavera et al 2004). Breathing pattern is assessed dynamically by analysis of box-pressure signals that increase and decrease with the respiratory cycle (Fig. 1) (Hofmann et al. 1999).

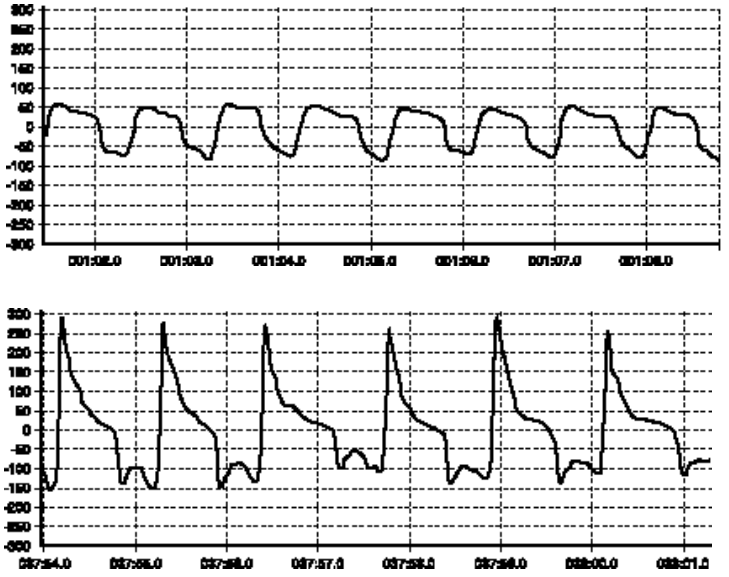

Fig. 1. An illustrative picture of box pressure signals obtained from a healthy cat by use of barometric whole body plethysmography.
1a - Signals obtained before aerosol exposure to carbachol.
1b - Signals obtained during peak bronchoconstriction after carbachol aerosol challenge.

Measurements derived by analysis of box-pressure signals change substantially with bronchoconstriction caused by different pharmacologic stimuli (e.g. histamine or metacholine challenge) (Chand et al. 1993; Hamelmann et al. 1997; Hofmann et al. 1999). Unfortunately, the pressure changes, occurring inside the closed plethysmograph chamber containing a breathing animal, are not only caused by the air flow during inspiration and expiration, but are also significantly influenced by changes in gas humidification and temperature as air moves between the box and the lungs. Since it is not possible to distinguish between these two sources of changes in box pressure, the usefulness of PENH in the assesment of lung function is accordingly to a certain degree limited (Jason et al. 2003; Adler et al 2004). Despite these limitations, BWBP is still

considered useful in clinical applications, especially because of its extreme non-invasiveness (Halley et al. 2004).

The purpose of our study was to investigate the effects of nebulized AMP on airflow limitation in healthy cats using barometric whole body plethysmography (BWBP), in comparison to the effects of carbachol. We hypothetized that carbachol, as directly acting agonist, would cause airflow limitation in healthy cats; in contrast, aerosol challenge with indirectly acting agent (AMP) should have no effects on healthy airways and cause no airflow limitation. Aerosol challenge with both bronchoprovocants might be useful in clinical practice as a non-invasive, easy to perform method for screening airway disease or monitoring the effect of drug therapy, especially in feline respiratory diseases of inflammatory origin.

Materials and Methods

Animals

Ten healthy Domestic shorthair cats ranging from 4 to 6 years (mean = 5.8) of age were included in the study. Three cats were castrated males and seven were neutered females. Animals were related and their body weight was 4 - 6 (mean = 4.86) kg. Cats in the study were kept as a colony of experimental animals at the Institute of Nutrition, Veterinary University Vienna, Austria. Their health status was regularly checked and revealed no history or clinical signs consistent with respiratory disease within 12 months of the onset of the study. All the animals were regularly vaccinated and dewormed. In all cats, a physical examination, CBC and biochemical blood analysis were performed prior to the first testing period, which revealed no abnormalities. Lateral and ventrodorsal radiographs of all the cats were examined. In nine animals, radiographs were considered as normal and in one cat a slightly increased interstitial pattern was found. All procedures were approved by the National Animal Health Care Authorities to fulfill the criteria concerning use of animals for experimental studies.

Procedure

We used a similar procedure for barometric whole body plethysmography (BWBP) to that described by Hoffman et al. (1999) in cats. Animals were placed in a BWBP chamber consisting of a plexiglass box with an inner volume of 38 l. A screen pneumotachograph was attached to the wall of the main chamber to permit dynamic assessment of pressure fluctuations in the chamber. One pole of a low-pressure differential transducer (SCXL004, Invensys Sensor Systems, Milpitas, Calif) (± 10 cm H_2O) was open to the main chamber and the other pole was open to a reference chamber equilibrated with atmospheric pressure by way of a small channel (1.5 mm; 67% decrease in pressure during a 10 second period). Continuous bias flow (4.8 l/min) was used to maintain the oxygen concentration while simultaneously preventing CO_2 from accumulating in the chamber (< 0.1% CO_2). Transduced signals were amplified, digitalized and sampled at 100 Hz by use of commercial software (Bio System XA, version 2.7 β, BUXCO Electronics Inc, Wilmington, NC) which also provided breath-by-breath analysis of waveforms. Inclusion criteria for waveforms that were analyzed included an inspiratory volume > 15 ml, inspiratory time (Ti) > 0.15 seconds and < 10 seconds, and a ratio of inspiratory volume to expiratory volume between 0.8 and 1.2 (Hirt et al. 2003).

Calibration of the chamber pressure signal was performed dynamically by injecting 50 ml of room air via syringe into the main chamber of the BWBP and integration of the area under the resulting flow curve. Pressure signals were analyzed by computer software to obtain values for different respiratory variables, especially enhanced pause (PENH). Enhanced pause is a unitless variable derived from observations that relaxation time (time from peak expiratory pressure in chamber to a value that is 30% of that peak) decreases and peak expiratory flow/peak inspiratory flow (i.e, ratio of peak chamber pressure during expiration to peak chamber pressure during inspiration) increases during bronchoconstriction in cats (Hofmann et al. 1999), guinea pigs (Chand et al. 1993) and mice (Hamelmann et al. 1997). Values for PENH were calculated as follows: $PENH = ([Te/RT] - 1) \times (PEF/PIF)$, where Te means expiratory time, RT relaxation time, PEF peak expiratory flow and PIF peak inspiratory flow. The protocol included measurement of PENH, tidal volume (TV) and respiratory rate (RR) before and after aerosol administration of vehicle and bronchoconstrictor agonists. Each animal was allowed to acclimate to the environment of the plethysmograph box for 3-5 minutes before each measurement. Firstly, measurement without nebulization was performed to obtain baseline values; this was subsequently followed by nebulization of 0.9 % saline (post-saline challenge) and finally by nebulization of increasing concentrations of the respective bronchoprovocative agonist. Carbachol (Carbachol, Fluka Chemie Gmbh, Buchs, Switzerland) to be nebulized was prepared at concentrations of 0.05, 0.1, 0.2, 0.5, 1, 2, 5 mg/ml (Hirt et al. 2003) and AMP (Adenosine 5'-monophosphate disodium salt, Fluka Chemie Gmbh, Buchs, Switzerland) at concentrations of 0.1, 1, 10, 100 and 500 mg/ml (Marks et al. 1996; Currie et al. 2003). Stock solutions were obtained by solving the dry powder in 0.9% saline. Dilutions were prepared with 0.9% saline and stored at a temperature of 4 °C during the whole testing period (Martínez-García et al. 2002).

PENH was used as a function of the increasing concentrations of bronchoprovocative agonists, to characterize airway responsiveness as a concentration-dependent response. Aerosol administration was performed through

a valved opening in the chamber by use of a jet nebulizer driven by a compressor (Pari Master, PARI Gmbh, Starnberg, Germany), which produced particles with a diameter of 2-3 mm on average. Aerosol administration of saline and the increasing concentrations of the different bronchoprovocative agents was performed for 1 min each, followed by a 7-min period of data acquisition, which also facilitated clearance of bronchoprovocative agent from the box. The peak value for PENH after each dose of agonist was the highest mean value for 10 consecutive breaths during the 7-min observation period. When the peak value for PENH exceeded 300% of postsaline challenge value (ie, 3 times the post-saline value) for > 10 consecutive breaths and substantial changes in respiratory pattern (e.g. increased expiratory effort) were observed clinically, additional aerosol administrations were not performed. The provocative concentration of the agonist that increased PENH to 300% of post-saline value (PCPENH300) was obtained by interpolation of the concentration-response curve between the final 2 doses of provocative agonist (Hirt et al 2003).

In order to assess the inter-day repeatability of measurements, a second bronchoprovocation with each agent was performed. Subsequent bronchoprovocation tests in individual animals were performed in 2-3 day intervals to exclude influences of prior nebulization. Cats were tested throughout the day always in the same order. Challenges with described bronchoprovocants were performed in the following order: day 0 - first carbachol challenge, day 2 - first AMP challenge, day 4 - second carbachol challenge, day 7 - second AMP challenge.

Statistical analysis

Data were statistically analyzed by ANOVA for repeated measurements using SPSS 11.5 for Windows to describe the inter-day repeatability of PCPENH300 values obtained by repeated nebulization of carbachol.

Results

For carbachol, PCPENH300 was reached in all cats and correlated significantly between days (mean ± SD; 0.54 ± 0.42 mg/ml and 0.64 ± 0.45 mg/ml respectively; r = 0.58, $p < 0.05$; Fig. 2). Tidal volume measured after saline and the highest concentration of carbachol being nebulized was 31.64 ± 11.11 ml and 59.71 ± 32.25 ml (mean ± SD) for the first carbachol challenge, 28.82 ± 23.63 ml and 86.00 ± 38.36 ml for the second carbachol challenge. Respiratory rate received after nebulisation of saline and the highest concentration of agonist was 49 ± 17 and 37 ± 12 breaths/minute (mean ± SD) for the first carbachol challenge.

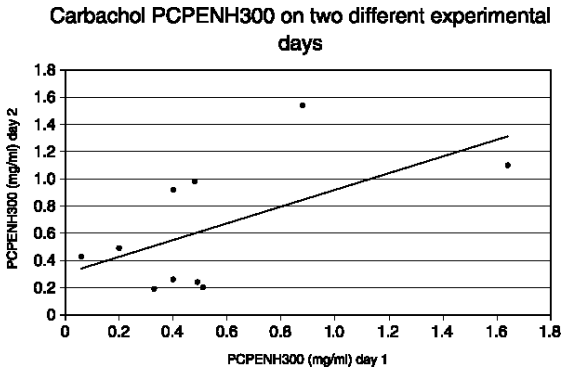

Fig. 2. The effects of nebulized carbachol on airflow limitation in the airways of 10 healthy Domestic shorthair cats by the use of barometric whole body plethysmography was investigated. Plot of carbachol PCPENH300 values (mean ± SD; 0.54 ± 0.42 mg/ml and 0.64 ± 0.45 mg/ml, respectively; r = 0.58, $p < 0.05$) obtained on two different experimental days. Each point represents one cat. Good agreement of measurements can be seen.

In contrast, we found no reaction to AMP even with the highest concentration nebulized during both measurements. At baseline, mean ± SD PENH was 0.47 ± 0.18 and 0.58 ± 0.24 (measurements 1 and 2), whereas PENH after 500 mg/ml AMP was 0.46 ± 0.20 and 0.71 ± 0.37. Values of TV mesured after saline and 500 mg/ml AMP nebulisation were (when expressed as mean ± SD breath/minute) 32.82 ± 24.85 and 31.90 ± 17.28 ml for the first AMP challenge, 49.44 ± 49.39 and 35.73 ± 13.24 ml for the second AMP challenge.

Respiratory rate measured after saline and 500 mg/ml AMP nebulisation were (when expressed as mean ± SD breaths/minute) 46 ± 15 and 47 ± 14 breaths/ minute for the first AMP challenge, 54 ± 14 and 49 ± 13 breaths/ minute for the second AMP challenge.

All bronchoprovocation tests were well tolerated by the cats.

Discussion

In this study, airway responsiveness to aerosol administration of carbachol and AMP in healthy cats was investigated. Using carbachol - known to be a directly acting bronchoprovocative agonist - an increase of PENH more than 300% over the baseline was obtained in all cats of this study. This observation was confirmed by repeated measurements and carbachol challenge was found to be a reliable and repeatable procedure. There was no increase over post-saline challenge PENH when AMP as an indirect agonist was nebulized. Bronchoprovocative challenge with both agents was considered safe. Despite the described limitations of BWBP (Jason et al. 2003; Adler et al. 2004), bronchoprovocation testing with carbachol can still be considered in clinical practice as an easy to perform, non-invasive method, which can be used not just for diagnostic screening, but also to monitor the effect of drug therapy in inflammatory respiratory diseases in cats. Adenosine 5'- monophosphate nebulization in cats with respiratory disease needs to be investigated to describe the clinical relevance of this bronchoprovocative agent.

Barometric whole body plethysmography (BWBP), as a non-invasive tool that allows detection of airflow limitations in cats, was introduced by Hoffman and coworkers already in 1999. Indeed it has been demonstrated in cats and other species that enhanced pause (PENH), an index of airflow limitation, can be used for the detection and quantification of obstruction of the lower airways (Hamelmann et al.1997; Hofmann et al. 1999; Hirt et al. 2003; Halley et al. 2004). Lately, studies describing uncertainity of this method have been published. In comparison with other more or less experimental methods, such as measurement of input impedance in anesthetized paralyzed and tracheostomized animals or other techniques describing changes in pulmonary resistance and dynamic compliance, BWBP is considered less reliable (Jason et al. 2003; Adler et al. 2004). The major problems are changes of pressure within the box of BWBP caused by increasing gas humidification and temperature as the animal breathes. Since it is not possible to estimate the influence of these two sources of changes in box pressure, the usefulness of PENH in the assesment of lung function is accordingly to a certain degree limited (Jason et al. 2003; Adler et al. 2004). Since the previously described more precise methods evaluating lung function require general anesthesia, they are often precluded from being applied to small animal clinical patients for repeated routine diagnostic purposes. BWBP, including measurement of airway responsiveness, is considered a promising, easy to perform method, which, despite its limitations, could add significantly to routine diagnostics in various lower airway diseases (Halley et al. 2004).

In the cats of this study, we applied a BWBP and bronchoprovocation procedure similar to that which has been described by Hoffman et al. (1999). Similarly to previous studies, an increase of PENH more than 300% over the baseline was obtained in all the cats after nebulization of increasing concentrations of carbachol (Hoffman et al. 1999; Hirt et al 2003). In our study, we did not find significant differences between PCPENH300 values for carbachol when measurements were repeatedly performed in individual animals. Carbachol has been shown to be a reliable bronchoprovocative agent for airway responsiveness testing in cats.

Tidal volume and respiratory rate, which are other important variables, were measured during bronchoprovocation testing. Comparison between post-saline TV values, and TV values in bronchoconstriction may not be valid, since TV measurements in

bronchoconstriction have been reported to be erroneously increased (Ingram and Schilder 1966). On the other hand, a drop in RR, when there is airflow limitation, is an important finding showing the increase of the expiratory effort which leads to prolongation of expiratory time and finally to a decrease in RR.

Adenosine is a purine nucleoside which may be generated in allergic inflammatory conditions upon appropriate stimulation. Once produced, adenosine is able to promote a large variety of responses in the airways, such as bronchoconstriction, plasma exsudation and increased bronchial blood flow. Furthermore, as a paracrine mediator, it contributes to various aspects of the inflammatory process (Polosa and Holgate 1997). Experimentally, adenosine was able to increase the contractile responses of canine airway smooth muscle induced by histamine (Sakai et al. 1989). Similar effects can be observed after inhalation of adenosine by subjects with lower airway disease (Rutgers et al. 1999; Rutgers et al. 2000). Cushley and co-workers (1983) first reported that adenosine provoked concentration-dependent bronchoconstriction when administered by inhalation to asthmatic subjects but not to healthy volunteers. In addition, inhalation of its related nucleotide, adenosine 5'- monophasphate, produces an almost identical effect on the airways, as it is dephosphorylated to yield adenosine (Polosa and Holgate 1997). Several other experiments demonstrated that aerosol administration of increasing concentrations of AMP caused no or a very weak bronchoconstrictor response in healthy mice, Brown Norway rats, guinea pigs or rabbits (Hannon et al. 2001; Fan and Mustafa 2002). Also in our study, as was hypothetized, we did not observe significant differences between post-saline PENH values and values after nebulization of AMP even in the highest concentration. This can be best explained by AMP's proposed mechanism of action. The occurrence of high numbers of AMP effector cells, such as mast cells, is not expected in the lower airways of healthy subjects. This fact supports the idea that indirectly acting agents (e.g. AMP) for testing airway hyperresponsiveness could be of greater value in the diagnosis of lower airway inflammatory diseases, since they might reflect the degree of inflammation more specifically than directly acting stimuli (Polosa and Holgate 1997; Van Schoor et al. 2000; Polosa et al. 2002).

In the current study we investigated differences in airway responsiveness to the two different bronchoprovocative agonists carbachol and AMP in healthy cats with the use of BWBP. Carbachol, a directly acting bronchoprovocative agonist, increased PENH to more than 300% over the baseline in all animals and was found to be reliable in causing reproducible airway responses when used as aerosol. As we hypothetized, there was no increase over baseline PENH with the indirect bronchoconstrictive agent AMP in these healthy cats. The use of AMP challenge in cats requires further research to evaluate the usefulness of this bronchoprovocative agent as a diagnostic tool in feline lower airway disease. Even if obvious limitations of BWBP have been described, still this extremely non-invasive method can be of value when used as a screening diagnostic method for certain inflammatory respiratory diseases, and for evaluation of the efficacy of drug therapy in cats.

Bronchoprovokace aerosolem adenosin 5'- monofosfátu nezpůsobuje u zdravých koček omezení průchodnosti dýchacích cest

Cílem naší studie bylo porovnat vliv adenosin 5'- monofosfátu a karbacholu na průchodnost dýchacích cest u zdravých koček při využití celotělové barometrické pletysmografie. Studie byla provedena na deseti zdravých evropských krátkosrstých kočkách ve věku 4 až 6 let. Každá kočka byla umístěna do plexisklové komory pletysmografu o objemu 38 l. Následně byly zaznamenávány změny tlaku uvnitř komory, a to nejprve bez nebulizace a poté po nebulizaci vehikula s obsahem karbacholu nebo adenosin 5'- monofosfátu ve stoupající koncentraci. Reakce dýchacích cest byla sledována jako

zvýšení tzv. prodloužené pauzy (enhanced pause, PENH), proměnné bez jednotky, kterou lze odvodit z pletysmografické křivky měnící se v závislosti na stupni omezení průchodnosti dýchacích cest. Bronchoprovokace byla ukončena, pokud určitá koncentrace agonisty (tzv. provokační koncentrace, PCPENH300) zvýšila PENH o 300 % hodnoty naměřené po nebulizaci NaCl. Za účelem potvrzení opakovatelnosti měření byly bronchoprovokační zkoušky oběma agonisty prováděny opakovaně v intervalu 2-3 dnů. Při nebulizaci karbacholu bylo u všech koček dosaženo omezení průchodnosti dýchacích cest a zjištěna signifikantní korelace hodnot PCPENH300 získaných při opakovaném měření (průměr ± směrodatná odchylka; 0.54 ± 0.42 mg/ml a 0.64 ± 0.45 mg/ml; $r = 0.58$, $p < 0.05$). Na rozdíl od karbacholu, nebulizace adenosin 5'- monofosfátu, včetně nejvyšší možné koncentrace, opakovaně nevyvolala reakci dýchacích cest. Po nebulizaci NaCl byly získány hodnoty PENH (vyjádřeno jako průměr ± směrodatná odchylka) 0.47 ± 0.18 a 0.58 ± 0.24 (měření 1 a 2), a po nebulzaci 500 mg/ml adenosin 5'- monofosfátu hodnoty 0.46 ± 0.20 a 0.71 ± 0.37. Veškeré bronchoprovokační zkoušky byly zvířaty velmi dobře tolerovány. Dýchací cesty zdravých koček nereagují na inhalaci adenosin 5'- monofosfátu. Toto zjištění je v souladu se studiemi prováděnými u lidí a také s naší studií, kdy u zdravých psů neměla inhalace adenosin 5'- monofosfátu žádný účinek, avšak u psů s vyvolanou akutní bronchitidou vedla k omezení průchodnosti dýchacích cest. Pro zhodnocení významu bronchoprovokačních testů s využitím adenosin 5'- monofosfátu u koček je nezbytný další výzkum zaměřený na účinky tohoto agonisty u koček se spontánním onemocněním dolních cest dýchacích.

Acknowledgements

The authors wish to express their thanks to Prof. Juergen Zentek from the Institute of Nutrition, Veterinary University Vienna, Austria for the possibility to use the experimental animals of the Institute.

References

ADLER A, CIESLEWICZ G, IRVIN CG 2004: Unrestrained plethysmography is an unreliable measure of airway responsiveness in BALB/c and C57BL/6 mice. J Appl Physiol **97**: 286-292

CHAND N, NOLAN K, PILLAR J, et al. 1993: Aeroallergen-induced dyspnea in freely moving guinea pigs: quantitative measurement of bias flow ventilated whole body plethysmography. Allergy **48**: 230-235

CURRIE GP, JACKSON CM, LEE DKC, et al 2003: Allergen sensitisation and bronchial hyperresponsiveness to adenosine monophosphate in asthmatic pacients. Clin Exp Allergy **33**: 1405-1408

CUSHLEY MJ, TATTERSFIELD AE, HOLGATE ST 1983: Inhaled adenosine and guanosine on airway resistance in normal and asthmatic subjects. Br J Clin Pharmacol **15**: 161-165

FAN M, MUSTAFA SJ 2002: Adenosine-mediated bronchoconstriction and lung inflammation in an allergic mouse model. Pulm Pharmacol Ther **15**: 147-155

HALLEY DJ, KIRSCHVINK NA, VINCKE GC et al 2004: Whole body barometric plethysmography: a screening method to investigate airway reactivity and acute lung injuries in freely moving pigs. The Veterinary Journal **168(3)**: 276-284

HAMELMANN E, SCHWARZE J, TAKEDA K et al 1997: Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. Am J Respir Crit Care Med **156**: 766-775

HANNON JP, TIGANI B, WILLIAMS I et al 2001: Mechanism of airway hyperresponsiveness to adenosine induced by allergen challenge in actively sensitized Brown Norway rats. Brit J Pharmacol **132**: 1509-1523

HIRT RA, DEDERICHS D, BOEHLER A et al 2003: Relationship of age, sex, body weight, and hematologic and respiratory variables with airway reactivity in adult cats. Am J Vet Res **64**: 26-31

HOFFMAN AM, DUPA N, CIMETTI L 1999: Airway reactivity measured by barometric whole-body plethysmography in healthy cats. Am J Vet Res **60**: 1487-1492

INGRAM RH Jr, SCHILDER DP 1966: Effects of gas compression on pulmonary pressure, flow and volume relationship. J Appl Physiol **21**: 1821-1826

JASON HT, BATES T, IRVIN CG 2003: Measuring lung function in mice: the phenotyping uncertainity principle. J Appl Physiol **94**: 1297-1306

MARKS GB, YATES DH, SIST M et al 1996: Respiratory sensation during bronchial challenge testing with metacholine, sodium metabisulphite, and adenosine monophosphate. Thorax **51**: 793- 798

MARTÍNEZ-GARCÍA MA, PERPINÁ-TORDERA M, VILA V et al 2002: Analysis of the stability of stored adenosine 5'- monophosphate used for bronchoprovocation. Pulm Pharmacol Ther **15**: 157-160

POLOSA R, HOLGATE ST 1997: Adenosine bronchoprovocation: a promising marker of allergic inflammation in asthma? Thorax **52**: 919-923

POLOSA R, RORKE S, HOLGATE ST 2002: Evolving concepts on the value of adenosine hyperresponsiveness in asthma and chronic obstructive pulmonary disease. Thorax **57**: 649-654

RUTGERS SR, KOETR GH, VAN DEN MARK TW et al 1999: Protective effect of oral terfenadine and not inhaled ipratropium on adenosine 5'- monophosphate induced bronchoconstriction in patients with COPD. Clin Exp Allergy **29**: 1287-1292

RUTGERS SR, TIMENS W, TZANAKIS N et al 2000: Airway inflammation and hyperresponsiveness to adenosine 5'- monophosphate in chronic obstructive pulmonary disease. Clin Exp Allergy **30**: 657-662

SAKAI N, TAMAOKI J, KOBAYASHI K et al 1989: Adenosine potentiates neurally- and histamine- induced contraction of canine airway smooth muscle. Int Arch Allergy Appl Immunol **90(3)**: 280-284

TALAVERA J, BILLEN F, GUSTIN P et al 2004: Whole-body plethysmography in healthy dogs: repeatability and measurement of airway responsiveness. Proceedings. 22th Annual ACVIM Forum 2004.

THEODOROU A, WEGER N, KUNKE K et al 1997: Ragweed sensitization alters pulmonary vascular responses to bronchoprovocation in beagle dogs. J Appl Physiol **83(3)**: 912- 917

VAN DEN BERGE M, MEIJER RJ, KERSTJENS HAM et al 2001: PC_{20} adenosine 5'-monophosphate is more closely associated with airway inflammation in asthma than PC_{20} metacholine. Am J Respir Crit Care Med **163**: 1546-1550

VAN SCHOOR J, JOOS GF, PAUWELS RA 2000: Indirect bronchial hyper-responsiveness in asthma: mechanisms, pharmacology and implications for clinical research. Eur Respir J **16**: 514-533

ACTA VET. BRNO 2006, 75: 337-341; doi:10.2754/avb200675030337

Suppressive Subtraction Hybridization on Stimulated Primary Horse Macrophages

J. MATIAŠOVIC [1,2], P. KRÁLÍK [1,2], L. LEVÁ [2], M. FALDYNA [2], P. HOŘÍN [1]

[1]Institute of Genetics, University of Veterinary and Pharmaceutical Sciences Brno
[2]Veterinary Research Institute Brno

Received August 2, 2005
Accepted April 13, 2006

Abstract

Matiašovic J., P. Kralik, L. Levá, M. Faldyna, P. Hořín: Suppressive Subtraction Hybridization on Stimulated Primary Horse Macrophages. Acta Vet Brno 75, 2006: 337-341.

To study genes potentially involved in genetic resistance to infectious diseases in the horse, suppressive subtraction hybridization was used to identify genes expressed in primary horse macrophages after their stimulation with *E. coli*. Overnight culture of blood monocyte-derived macrophage cells was stimulated with *E. coli* K12 in ratio 40 *E. coli* cells to one macrophage cell. After 4 hours of incubation, non-phagocyted bacteria were washed away. Following next 20 hour incubation in MEM alpha containing 5 μg of gentamycin in 1 ml of media, mRNA was isolated and used in Clontech PCR-Select cDNA Subtraction Kit. Expression of several known horse genes, as well as some new ESTs (expressed sequence tags) showing sequence similarity with immunity-related genes from other species was identified.

SSH, horse, monocyte, immunity

Several methods can be used to identify differentially expressed mRNAs. Differential display, subtractive hybridization and serial analysis of gene expression (SAGE) are widely used methods. SAGE is probably the most powerful, and not only qualitative, but also a quantitative method (Pylouster et al. 2005). However, this method is also very expensive, due to the number of sequencing reactions needed. Differential display analysis uses acrylamide gels for separation and identification of ESTs (expressed sequence tags). The gel-based separation and subsequent DNA isolation represents an inconvenience of this procedure. Usually tens of ESTs are identified (Chakrabarti et al. 2002; Lee et al. 2003). Subtractive hybridization in combination with suppressive PCR can trap differentially expressed genes without needing physical separation of products of hybridization (ssDNA and dsDNA) (Diatchenko et al. 1996). The result of the whole procedure is a pool of PCR products representing ESTs.

Macrophage reactions to pathogens are mediated not only by the type of antigen, but they are also actively influenced by the pathogen. For example, *M. tuberculosis* decreases production of IL-12 in human primary monocyte-derived macrophages, while *E. coli* cells were found to be an activator of a wide range of genes in stimulated human primary monocyte-derived macrophages (Nau et al. 2002).

The number of known immune-related genes in horse is limited. The objective of this work was to identify differentially expressed sequence tags (ESTs) in stimulated and/or non-stimulated horse macrophage cells.

Materials and Methods

Monocyte derived macrophage cells: a modified protocol of Raabe et al. (1998) was used. Briefly: 0.5 l of whole blood from a healthy horse was centrifuged on Histopaque (Sigma-Aldrich, Germany)/Telebrix (Léčiva a.s., Czech Republic) mixture with density of 1.098. Peripheral blood mononuclear cells were placed on plastic dishes coated with 2% gelatine (Sigma-Aldrich, Germany) and incubated in MEM alpha (Sigma-Aldrich, Germany) supplemented with glutamine (Sigma- Aldrich, Germany), 10% horse sera (Sigma-Aldrich, Germany) and 100 U/100 μg/ml of penicillin/streptomycin (PAA Laboratories, Austria). After 3 hours of incubating at 37 °C with 5% CO_2, nonadherent cells were washed away. Adhered monocyte cells were incubated overnight.

Address for correspondence:
MVDr. Ján Matiašovic, Ph.D.
Veterinary Research Institute
Department of Immunology
Hudcova 70, 621 00 Brno, Czech Republic

Phone: +420 533 331 317
Fax: +420 541 211 229
E-mail: matiasovic@vri.cz
http://www.vfu.cz/acta-vet/actavet.htm

Stimulation: Overnight culture of monocyte-derived-macrophage cells were stimulated with viable *E. coli* K12 in ratio of 40 bacteria to 1 macrophage cell in media without antibiotics. After four hours, cells were gently washed and incubated for 20 hours with media containing 5 μg/ml of gentamycin. After that, cells were lysed directly on dish with TRIReagent (Sigma-Aldrich, Germany). Changes in cell size and granularity were measured on flow cytometer (FACSCalibur, Becton Dickinson, NJ USA). Non-stimulated control cells were treated in the same conditions, except for the addition of *E. coli* into media.

mRNA isolation: mRNA was isolated from total RNA using Oligotex mRNA Mini Kit (Qiagen, CA USA).

Suppressive subtraction hybridization (SSH): isolated mRNA from stimulated and/or non-stimulated cells was used in suppressive subtraction hybridization. A Clontech PCR-Select cDNA Subtraction Kit (BD Biosciences, NJ USA) was used for this purpose. According to the manual, the forward and reverse subtracted probes were made for differential screening of subtracted products.

Differential screening of subtracted products: subtracted products were resolved on 1% agarose gel. DNA was blotted on Hybond N+ nylon membrane (Amersham Biosciences, Sweden) and hybridized with forward and/or reverse subtracted probes using AlkPhos Direct Labeling and Detection System with CDP-star (Amersham Biosciences, Sweden).

Cloning and sequencing: SSH products were cloned into pDrive vector (PCR Cloning kit, Qiagen, CA USA) and pCRXL vector (TOPO XL Cloning kit, Invitrogen, CA USA). Plasmids were sequenced in MWG company (Germany), while PCR products on ABI 310 (Applied Biosystems, CA USA) .

Blast analysis: searches of obtained ESTs against sequences deposited to GeneBank/EMBL/DDBJ databases were performed with blastn software (Altschul et al. 1997, http://www.ncbi.nlm.nih.gov/BLAST/) using default parameters (low-complexity filter, word size 11, expect statistical significance threshold 10). ESTs with no hit to GeneBank/EMBL/DDBJ databases using blastn (ESTs DQ138065, DQ138061, DQ138064, DQ138063) were searched against human, mouse, cow, pig and dog genomes using cross-species megablast (expect statistical significance threshold 10, http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast).

Results and Discussion

Changes in granularity and size of cells were observed by FACS after stimulation of primary horse macrophages with viable *E. coli* (Fig. 1). Using SSH, 7 fragments were

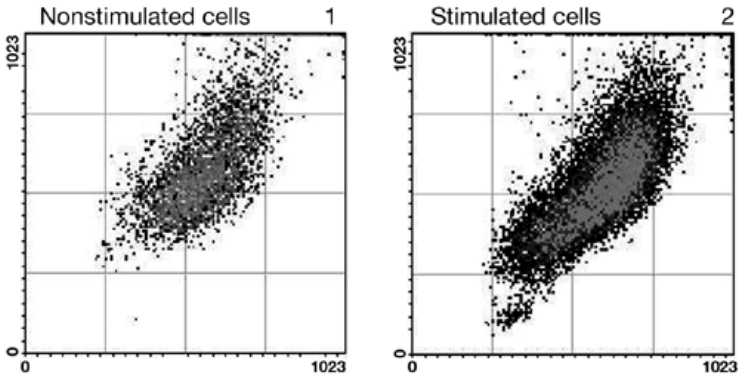

Fig.1. Side scatter (horizontal scale) represents cell granularity, forward scatter (vertical scale) represents cell size.

obtained from the cells after stimulation with *E. coli*, while 17 fragments were obtained from non-stimulated macrophages. Another 6 fragments were isolated from stimulated cells following a second independent SSH. This is a relatively low number as compared to the number of genes known to be involved in the activation program of human macrophages (Nau et al. 2002). This is probably due to a low efficiency of the procedure used. On the other hand, the number of genes differentially expressed after macrophage activation may be biased by macrophage activation caused by cell adherence on plastic dish, regardless of the presence of *E. coli* cells. Bands representing SSH products were cut out of the gel and

reamplified for sequencing. As some bands could not be reamplified successfully, SSH products were cloned into pDrive and pCRXL vectors, and clones with different length of inserts were sequenced. Some genes were present in more than one fragment. The genes identified are summarized in Table 1. Four of 7 new sequences from stimulated cells represent putative horse chemokines, based on their homology with nucleotide sequences

Table1. ESTs identified in macrophage by SSH

Products from stimulated cells		
EST similar to	% of identity	accession number
equine MGSA AF053497	98	
equine IL1beta ECU92481	98	
equine G-CSF AF503365	99	
human THBS1 NM_003246	96	DQ138067
human CXC ligand 1 NM_001511	81	DQ138062
human CXC ligand 5 BC008376	82	DQ138068
porcine AMCF II NM_213876	87	DQ138066
bovine GCP2 AF149249	88	DQ138069
fragment 48 from SSH AY246812	100	
uknown sequence H9		DQ138065
unknown sequence S7		DQ138061
Products from non-stimulated cells		
EST similar to	% of identity	accession number
equine MGSA AF053497	98	
equine IL1beta ECU92481	98	
equine PAI 2 AF508790	99	
equine satellite TKY504 AF508790	94	
equine clone 37c AY029358	90	
equine clone CH241-77F8 AC153071	85	
bovine genomic contig NW_351856 and/or human genomic contig NT_010393	78* 76*	DQ138064
unknown sequence N11		DQ138063

*similarity found using cross-species megablast

from other species. It corresponds well to their function in macrophages stimulated with bacteria, which is also the case of the putative horse thrombospondin 1 (Narizhneva et al. 2005). Another fragment is identical with the GenBank sequence AY246812 [product 48 from SSH from an interleukin 1b treated equine synovium, Takafuji et al. (2003), published only as an GenBank entry]. Two other fragments were not homologous to any

known sequences deposited in GenBank /EMBL/DDBJ using blastn, as well as to human, mouse, cow, pig and dog genomes using cross-species megablast. Presence of fragments containing non-coding sequences in products from non-stimulated cells was not anticipated (equine satellite TKY504 AF508790, equine clone 37c AY029358, equine clone CH241-77F8 AC153071, EST DQ138063 and EST DQ138064 similar to bovine genomic contig NW_351856 and/or human genomic contig NT_010393), as contaminating DNA should not be present in mRNA samples due to mRNA preparation by hybridization of polyA tails to immobilized oligo-dT. Despite the fact that the first SSH Southern-blot hybridization with forward and reverse subtracted probes showed differential expression of the SSH products, the MGSA (melanoma growth stimulatory analog) and IL1beta genes were found in products from non-stimulated cells from the first SSH, but also in products from stimulated cells at the second SSH, which is not in agreement with theoretical expectations. This may be considered as an example of false positive results representing a common problem of methods used for identification of differentially expressed genes (Chen et al. 2004). The results presented here thus showed that the efficiency and specificity of the used SSH protocol was not optimum. It seems that efficiency of the SSH procedure is very sensitive to reaction conditions and can be used only as a preparative, not analytical tool. However, obtaining 9 newly identified sequences showed that SSH may be a feasible genomic approach for identifying ESTs in horse macrophages, including the identification of so far unknown sequences. In summary, this approach showed that under the experimental conditions used in this study, macrophages express genes involved in chemokine signalling.

Použití supresní subtrakční hybridizace na stimulované primární makrofágy koně

Ke studiu genů s předpokládaným vlivem na genetickou rezistenci k infekčním nemocem koně jsme použili supresní subtrakční hybridizaci jako nástroj k identifikaci genů exprimovaných v primárních koňských makrofázích po jejich stimulaci *E. coli*. Kultura makrofágů odvozených z krevních monocytů byla stimulována *E. coli* K12 v poměru 40 bakteriálních buněk na 1 buňku makrofága. Nefagocytované bakterie byly po 4 hodinách kultivace odmyty. Po dalších 20 hodinách kultivace v mediu MEM alfa s přídavkem 5 mg gentamycinu na 1 ml media, byla izolována RNA a použita pro supresní subtrakční hybridizaci (Clontech PCR- Select cDNA Subtraction Kit). Kromě několika u koně již známých genů byly nalezeny nové sekvence podobné genům imunitní odpovědi známých u jiných živočišných druhů.

Acknowledgement

The work was supported by the Czech Science foundation (projects 523/02/D126 and 523/02/1526) and Ministry of Agriculture of the Czech Republic (project 0002716201). Thanks to Dr. Rychlík and Mgr. Gregorová from Veterinary Research Institute for direct sequencing of PCR products.

References

ALTSCHUL SF, MADDEN TL, SCHÄFFER AA, ZHANG J, ZHANG Z, MILLER W, LIPMAN DJ 1997: Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, Nucleic Acids Res **25**: 3389-3402

DIATCHENKO L, LAU YF, CAMPBELL AP, CHENCHIK A, MOQADAM F, HUANG B, LUKYANOV S, LUKYANOV K, GURSKAYA N, SVERDLOV ED, SIEBERT PD 1996: Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci USA **93**: 6025-30

CHAKRABARTI R, ROBLES LD, GIBSON J, MUROSKI M 2002: Profiling of differential expression of messenger RNA in normal, benign, and metastatic prostate cell lines. Cancer Genet Cytogen **139**:115-25

CHEN Y, WANG B, WEINING S, DAGGARD G 2004: Anchor primer associated problems in differential display reverse transcription polymerase chain reaction. Anal Biochem **329**: 145-147

LEE SS, SEO HS, CHOI SJ, PARK HS, LEE JY, LEE KH, PARK JY 2003: Characterization of the two genes differentially expressed during development in human fetal astrocytes. Yonsei Med J **44**:1059-68

NARIZHNEVA NV, RAZORENOVA OV, PODREZ EA, CHEN J, CHANDRASEKHARAN UM, DICORLETO PE, PLOW EF, TOPOL EJ, BYZOVA TV 2005: Thrombospondin-1 up-regulates expression of cell adhesion molecules and promotes monocyte binding to endothelium. FASEB J **19**: 1158-60

NAU JG, RICHMOND JFL, SCHLESINGER A, JENNINGS EG, LANDER ES, YOUNG RA 2002: Human macrophage activation programs induced by bacterial pathogens. Proceedings of the National Academy of Sciences **99**: 1503- 1508

PYLOUSTER J, SENAMAUD-BEAUFORT C, SAISON-BEHMOARAS TE 2005: WEBSAGE: a web tool for visual analysis of differentially expressed human SAGE tags. Nucleic Acids Res **33** (Web Server issue): W693-5

RAABE MR, ISSEL CJ, MONTELARO RC 1998: Equine monocyte-derived macrophage cultures and their applications for infectivity and neutralization studies of equine infectious anemia virus. J Virol Methods **71**: 87-104

ACTA VET. BRNO 2006, 75: 343-353; doi:10.2754/avb200675030343

Effect of Isolation Techniques on Viability of Bovine Blood Neutrophils

P. SLÁMA[1], Z. SLÁDEK[1,2], D. RYŠÁNEK[2]

[1]Mendel University of Agriculture and Forestry in Brno, Czech Republic
[2]Veterinary Research Institute, Brno, Czech Republic

Received October 24, 2005
Accepted April 13, 2006

Abstract

Sláma P., Z. Sládek, D. Ryšánek: Effect of Isolation Techniques on Viability of Bovine Blood Neutrophils. Acta Vet Brno 2006, 75: 343-353.

The effect of selected isolation methods on the viability of neutrophil granulocytes (neutrophils) from the blood of healthy Holstein × Bohemian Red Pied crossbred heifers was evaluated. Two methods of neutrophil isolation were used: a) neutrophil isolation on the basis of hypotonic erythrocyte lysis (in two variants: after the erythrocyte lysis proper, the cells were centrifuged at either 200 *g* or 1000 *g*), and b) neutrophil isolation with FACS Lysing Solution as the lysing agent. The viability of the isolated neutrophils was evaluated on the basis of apoptosis and necrosis. The results obtained with flow cytometry (FCM) suggest that, from the isolation techniques used, the method based on FACS Lysing Solution impaired the neutrophil viability least. After the application of this method, 5.36 ± 2.15% of neutrophils were apoptotic and 0.51 ± 0.12% were necrotic. In contrast, when the hypotonic erythrocyte lysis was used, the proportion of apoptotic neutrophils amounted to 42.14 ± 7.12% and 49.00 ± 14.70%, respectively, and 41.12 ± 5.55% and 36.91 ± 24.38% respectively of necrotic neutrophils ($P < 0.01$). This was also confirmed by the light microscopy. After the isolation with FASC Lysing Solution, 1.92 ± 1.74% of neutrophils were apoptotic and 1.05 ± 0.76% were necrotic, as distinct from after the hypotonic erythrocyte lysis where 9.43 ± 3.69% of neutrophils were apoptotic and 12.67 ± 4.74% of necrotic after centrifugation at 200 *g*, while 12.60 ± 4.35 were apoptotic and 14.96 ± 12.64% were necrotic after centrifugation at 1000 *g*. It follows from the above-mentioned data that hypotonic lysis is not a suitable method for the isolation of neutrophils, as the method itself markedly affects cell viability.

Neutrophil, apoptosis, necrosis, phosphatidylserine, Annexin-V, propidium iodide, hypotonic erytrocyte lysis, FACS Lysing Solution, light microscopy, flow cytometry

Neutrophil granulocytes (neutrophils) constitute a significant component of the organism's non-specific defence system acting against bacterial infection, being the first line of cellular protection against invading microorganisms. After developing from the bone-marrow stem cells (Smith 2000), they are subsequently released into the blood stream where they circulate for only 6 - 10 hours (Kuijpers and Roos 2001). Following chemotactic signals, they migrate from the blood space into the tissues where they function as phagocytes (Paape et al. 2003). There the neutrophils are active for 2 - 6 days, then they undergo apoptosis and are removed by the tissue macrophages (Kuijpers and Roos 2001) irrespective of whether their phagocytic capacity was used or not (Junqueira et al. 1997). Because of their unique importance in the body, the neutrophils have become the subject of a great number of scientific papers (Paape et al. 2003).

It is obvious that the study of these blood elements *in vitro* depends on the creation of suitable conditions that include, in particular, the most gentle isolation and high purity of cells. For this reason, methods were developed that, by means of physical or biochemical procedures, enable a quick and effective separation of the blood neutrophils for *in vitro* studies. The following techniques can be used for the isolation of neutrophils from the blood: hypotonic erythrocyte lysis (Carlson and Kaneko 1973; Duque et al. 1985; Van Oostveldt et al. 1999), erythrocyte lysis by NH_4Cl (Roets et al. 1999; Van Oostveldt

Address for correspondence:
P. Sláma
Mendel University of Agriculture and Forestry in Brno
Zemědělská 1, 613 00 Brno
Czech Republic

Phone: +420 545 133 146
E-mail: xslama@node.mendelu.cz
http://www.vfu.cz/acta-vet/actavet.htm

et al. 1999), neutrophil adhesion on a microscopic slide (Smith and Rommel 1977), isolation of bovine neutrophil granulocytes from peripheral blood based on centrifugation in a discontinuous metrizamide gradient (Hallen-Sandgren and Bjork 1988) or by means of biomagnetic beads (Soltys et al. 1999), Percoll density gradient isolation (Boyum 1968; Van Oostveldt et al. 1999; Cowland and Borregaard 1999; Soltys et al. 1999), erythrocyte lysis with FACS Lysing Solution (Chen et al. 1996; Carulli et al. 1998; Tay et al. 1998; Hodge et al. 1999; Storie et al. 2003). It is evident from this short overview that the neutrophils are exposed to not only physical manipulation but also to non-physiological conditions during the isolation. This may result in unwanted effect on basic biological properties of isolated cells. Van Oostveldt et al. (1999) drew attention to this fact in the sphere of veterinary biology. They used hypotonic erythrocyte lysis for the isolation of neutrophils, and they detected early stages of neutrophil apoptosis using only flow cytometry with labelled Annexin-V and propidium iodide according to Vermes et al. (1995). This method appears as very advantageous for the analysis of blood elements when a large number of samples are processed. Its disadvantage is the poor detection of early stages of apoptosis, which cannot be morphologically differentiated from normal cells. Thus it does not comprise the whole population of apoptotic cells. If conventional haematological methods, such as blood smears and their analysis, particularly by light microscopy are used, these early apoptotic neutrophils appear "invisible". Apoptosis, however, is a highly dynamic process and therefore one can expect the occurrence of apoptotic neutrophils with fully developed morphological features, such as the nucleus chromatin condensation, cell shrinkage, zeiosis, etc. in the population of isolated cells (Arends et al. 1990; DelVecchio et al. 1993; Granville et al. 1998; Reed 2000). It is thus obvious that, in addition to biochemical methods, also morphological characteristics specific for apoptosis must be analysed for the evaluation of the effect of isolation methods on neutrophil viability. Until now, however, it has not been known whether the isolation techniques also result in morphological manifestations of neutrophil apoptosis.

For these reasons, it was the aim of this work to prove whether the techniques of erythrocyte lysis cause not only biochemical but also, and in particular, morphological changes typical of apoptosis and necrosis of bovine blood neutrophils.

Materials and Methods

Animals and experimental design

The experiments were carried out in duplicate in four clinically healthy Holstein × Bohemian Red Pied crossbred virgin heifers aged 16 to 18 months. They were used as blood neutrophil donors for studies of isolation techniques effect. The experiments were designed to monitor the effect of two isolation techniques, namely the isolation of neutrophils using hypotonic erythrocyte lysis, and isolation based on the use of the FACS Lysing Solution. Signs of neutrophil apoptosis and necrosis were detected by light microscopy and flow cytometry.

Blood sampling

The blood (150 ml) was drawn from the jugular vein into a sterile flask with an anticoagulant, namely Heparin (Léčiva a.s., Dolní Měcholupy, Czech Republic) 1500 I.U. in 15 ml of buffered saline (PBS) (Phosphate buffered saline - Sigma, Saint Louis, Missouri, USA).

Isolation of neutrophils

Hypotonic erythrocyte lysis (Carlson and Kaneko 1973): Heparinized blood was exposed to hypotonic conditions for 40 seconds. During this time, 50 ml of heparinized blood was mixed with 100 ml of redistilled water. Then 50 ml of 2.7% NaCl solution was added for the restoration of the standard osmotic pressure. This was followed by a 10-min centrifugation period, in which one of two variant techniques was always used: at the centrifugal force of 200 *g* - the "gentle" variant and at 1000 *g* - the "rough" variant). After the centrifugation, the supernatant was decanted and 50 ml of PBS were added. The procedure was then repeated starting from the erythrocyte lysis up to the centrifugation. In both variant techniques, the material was subsequently resuspended in PBS, centrifuged at 200 g again and resuspended in RPMI-1640 (Sigma, Saint Louis, Missouri, USA).

FACS Lysing Solution (Hodge et al. 1999): A blood sample (500 μl) was mixed with working solution of Annexin-V and PI and, after 15 min, it was kept in the dark at room temperature. After adding 2 ml of FACS Lysing Solution (Becton Dickinson Biosciences, San Jose, California, USA), the sample was resuspended and centrifuged

for 5 min at 500 *g*. Then the supernatant was decanted and 2 ml of PBS were added. New 5-min centrifugation at 500 *g* followed, the supernatant was removed and the sediment was resuspended in 500 μl of RPMI-1640.

Both techniques were carried out at laboratory temperature (20 to 23 °C).

Methods for detecting apoptosis and necrosis

Light microscopy: Smears of the cell suspension in RPMI were prepared by applying 10 μl of the suspension and 10 μl of autologous bovine serum. Slides stained by the Pappenheim method (May-Grünwald, Giemsa-Romanowski stain) were examined by light microscopy with oil immersion (Olympus BH2, Olympus Optical Co., LTD, Tokyo, Japan). Apoptosis and necrosis of isolated neutrophils were assessed by the enumeration of at least 200 neutrophils according to morphological features previously described (Sládek and Ryšánek 2000).

Flow cytometry: Apoptotic and necrotic neutrophils were analysed by FCM after simultaneous staining with Annexin-V labelled with fluorescein isothiocyanate (FITC) plus propidium iodide (PI) as described by Vermes et al. (1995). The commercial AnnexinV-FLUOS Staining Kit (Boehringer Mannheim, GmbH, Mannheim, Germany) was used according to the manufacturer's instructions in the procedure described below.

The cell suspension was adjusted to $1 \cdot 10^6$ per 1 ml in 100 ml of fresh incubation buffer containing FITC-Annexin-V and PI, and the suspension was analysed after 15-min incubation at room temperature by FCM (FACS Calibur apparatus, Beckton Dickinson, Mountain View, California, USA) by differentiating at least 10 000 cells. The neutrophil region was gated in accordance with procedure published Van Oostveldt et al. (1999). Dot plots were evaluated qualitatively and quantitatively using the WinMDI 2.8 software (Trotter 2000).

Statistics

The proportions of apoptotic and necrotic neutrophils from blood of the four heifers are shown as arithmetic means and standard deviations. The normality of a data distribution was proved by Kolmogorov test. The statistical significance of differences in the proportion of apoptotic and necrotic neutrophils were determined by the *t*-test. The data were processed by the STAT Plus software (Matoušková et al. 1992).

Results

Two methods of erythrocyte lysis were used in the study: hypotonic lysis in two variants differing in the centrifugal force (expressed in *g* - gravity force units) applied in the centrifugation after the erythrocyte lysis proper, namely 200 *g* and 1000 *g*, and erythrocyte lysis with FACS Lysing Solution. The analysis of neutrophils using FCM and morphological methods showed significant differences, particularly in the cell yield and in the proportion of apoptotic and necrotic cells detected.

Neutrophil yield

The FCM evaluation showed a statistically highly significant difference ($P < 0.01$) in the neutrophil yield (percentage of neutrophils in total leukocytes) between the method with FACS Lysing Solution (ca 30%) and the hypotonic lysis (ca 40%) (Table 1). In contrast, the light microscopy failed to show any significant difference in neutrophil yields between the above-stated methods (Table 2). The relatively low yield improved when the cell adhesion technique was applied (Table 5). The neutrophil yield was significantly higher on the adhesion assay slides (hypotonic lysis plus "gentle" centrifugation: 90.64 ± 4.49%, hypotonic lysis plus "rough" centrifugation: 90.49 ± 4.25%) than on the smears (hypotonic lysis plus "gentle" centrifugation: 33.25 ± 4.40%, hypotonic lysis plus "rough" centrifugation: 33.30 ± 13.86%) ($P < 0.01$).

In this evaluation of isolated neutrophils obtained by both methods, cells were analysed that showed biochemical and morphological features specific for apoptosis.

Biochemical and morphological features of apoptotic neutrophils

Apoptotic neutrophils were detected with both the FCM and the light microscopy methods.

In the dot plots obtained in FCM analysis after staining with Annexin-V plus PI, the neutrophils formed three subregions spaced in three quadrants: normal neutrophils (Annnexin-V negative/PI negative) took up the lower left quadrant, apoptotic neutrophils (Annexin-V positive/PI negative) took the lower right quadrant and the necrotic ones (Annexin-V positive/PI positive) were in the upper right quadrant (Fig. 1 a, b).

Table 1. Proportions of apoptosis and necrosis in bovine neutrophil granulocytes after isolation from blood. Flow cytometry (Annexin-V/propidium iodide).

	FACS Lysing Solution [a]		Centrifugation 200 *g* [b]		Centrifugation 1000 *g* [c]		Significance
	Arithmetic mean	S.D.	Arithmetic mean	S.D.	Arithmetic mean	S.D.	
Neutrophils [%]	32.13	3.63	42.39	4.24	38.37	4.14	++ a:b ++ a:c
Apoptotic neutrophils – An.-V+/PI- [%]	5.36	2.15	42.14	7.12	49.00	14.70	++ a:b ++ a:c
Necrotic neutrophils – An.-V+/PI+ [%]	0.51	0.12	41.12	5.55	36.91	24.38	++ a:b ++ a:c

+ $P < 0.05$; ++ $P < 0.01$
An.-V+: Annexin V positivity; PI-: propidium iodide negativity; PI+: propidium iodide positivity.
The proportions of apoptotic and necrotic neutrophils stated in the table were been calculated from the total count of neutrophils.

Table 2. Proportions of apoptosis and necrosis in bovine neutrophil granulocytes after isolation from blood. Light microscopy – smears.

	FACS Lysing Solution [a]		Centrifugation 200 *g* [b]		Centrifugation 1000 *g* [c]		Significance
	Arithmetic mean	S.D.	Arithmetic mean	S.D.	Arithmetic mean	S.D.	
Neutrophils [%]	36.27	4.19	33.25	4.40	33.30	13.86	-
Apoptotic neutrophils [%]	1.92	1.74	9.43	3.69	12.60	4.35	++ a:b ++ a:c
Necrotic neutrophils [%]	1.05	0.76	12.67	4.74	14.96	12.64	++ a:b + a:c

+ $P < 0.05$; ++ $P < 0.01$
The numbers of apoptotic and necrotic neutrophils stated in the table were calculated from the total count of neutrophils.

Table 3. Proportions of apoptosis and necrosis in bovine neutrophil granulocytes after isolation from blood. Light microscopy – adhesion slides.

	Centrifugation 200 *g* [b]		Centrifugation 1000 *g* [c]		Significance
	Arithmetic mean	S.D.	Arithmetic mean	S.D.	
Neutrophils [%]	90.64	4.49	90.49	4.25	-
Apoptotic neutrophils [%]	3.77	2.23	3.21	2.60	-
Necrotic neutrophils [%]	2.88	1.30	3.04	2.25	-

+ $P < 0.05$; ++ $P < 0.01$
Proportions of apoptotic and necrotic neutrophils shown in the table were calculated from the total count of neutrophils.

Detailed analysis of the suspension of isolated neutrophils in the light microscopy showed apoptotic neutrophils that occurred in three structurally different stages. These stages were

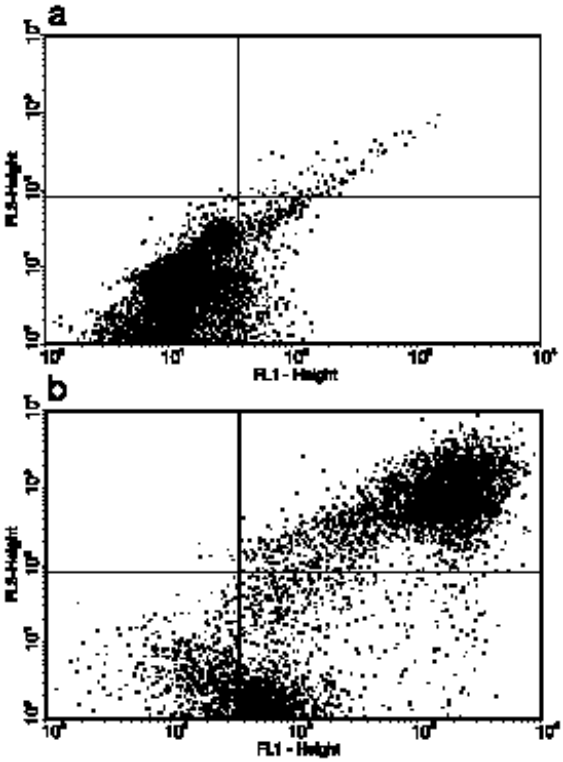

Fig. 1. After staining with Annexin-V plus propidium iodide, the neutrophils formed three subregions spaced in three quadrants in FCM dot-plots: normal neutrophils (An.-V-/PI-) took the lower left quadrant, apoptotic neutrophils (An.-V+/PI-) the lower right quadrant and the necrotic neutrophils (An.-V + /PI +) took the upper right quadrant. An.-V + : Annexin V positivity; PI-: propidium iodide negativity; PI + : propidium iodide positivity.
a: FACS Lysing Solution
b: hypotonic lysis

Table 4. The effect of adherence technique on the proportions of apoptosis in bovine blood neutrophils. Light microscopy.

	Adhesion slides [a]		Smears [b]		Significance
	Arithmetic mean	S.D.	Arithmetic mean	S.D.	
Centrifugation at 200 *g*	3.77	2.23	9.43	3.69	++ [a:b]
Centrifugation at 1000 *g*	3.21	2.60	12.60	4.35	++ [a:b]

+ $P < 0.05$; ++ $P < 0.01$
Proportions of apoptotic neutrophils (%) shown in the table were calculated from the total count of neutrophils.

karyopyknosis, zeiosis and the stage of apoptotic bodies. A reduction in nucleus size, chromatin condensation and the loss of pseudopodia were typical of karyopyknosis (Plate I, Fig. 2). Zeiosis was manifested as the blebbing of the cytoplasmic membrane; the cells took

Table 5. The effect of adherence technique on the yield of bovine blood neutrophils. Light microscopy.

	Adhesion slides [a]		Smears [b]		Significance
	Arithmetic mean	S.D.	Arithmetic mean	S.D.	
Centrifugation at 200 *g*	90.64	4.49	33.25	4.40	++ [a:b]
Centrifugation at 1000 *g*	90.49	4.25	33.30	13.86	++ [a:b]

+ $P < 0.05$; ++ $P < 0.01$
Proportions of neutrophils (%) shown in the table were calculated from the total count of leukocytes.

a foamy or honeycomb appearance (Plate I, Fig. 3). The next stage was manifested as the formation of cell fragments of various sizes: the apoptotic bodies (Fig. 4).

On the basis of the abovementioned biochemical and morphological features, the proportions of apoptotic neutrophils were determined using FACS and light microscopy in the particular isolation methods used.

Flow cytometry analysis

The results obtained in FCM make it obvious that the method with the FACS Lysing Solution affected the neutrophil viability to the least extent when the afore-mentioned isolation techniques are considered. The FCM method resulted in 5.36 ± 2.15% of apoptotic and 0.51 ± 0.12% of necrotic neutrophils, which means that Annexin-V positivity was established in a total of 5.87% isolated neutrophils. When the hypotonic-lysis method was used (Table 1), 83.26% and 85.91% Annexin-V positive neutrophils were found after the "gentle" and after the "rough" centrifugation, respectively.

Light microscopy analysis

When the hypotonic lysis and the FACS Lysing Solution methods were compared, it was established that the FACS Lysing Solution method impaired the neutrophil viability the least (1.92 ± 1.74% of apoptotic and 1.05 ± 0.76% of necrotic neutrophils). Hypotonic lysis and "gentle" centrifugation showed a more marked damage to neutrophil viability (9.43 ± 3.69% of apoptotic and 12.67 ± 4.74% of necrotic cells), while the "rough" centrifugation resulted in the highest proportion of apoptotic and necrotic neutrophils (12.60 ± 4.35% and 14.96 ± 12.64% respectively) (Table 2).

Surprisingly, statistically significant differences in the proportion of apoptotic neutrophils on the smears as compared with the adherent fraction were observed ($P < 0.01$). As the Tables 3 and 4 show, the proportion of apoptotic neutrophils was 3 - 4 times higher on the smears than on the adhesion slides.

A comparison of biochemical and morphological features of apoptosis in bovine blood neutrophils in the flow cytometer and in the light microscope is shown in Table 6.

Discussion

The objective of this work was to establish whether the technique of erythrocyte lysis causes biochemical and morphological changes typical of neutrophil apoptosis and necrosis. Methods of FCM and light microscopy were used for this purpose.

For the isolation of the blood neutrophils, the technique specially developed for FCM was used, namely the method with FACS Lysing Solution, in addition to the erythrocyte

Table 6. Comparison of biochemical and morphological features of bovine blood neutrophils apoptosis in a flow cytometry and in a light microscopy (smears).

	Flow cytometer [a]		Light microscopy [b]		Significance
	Arithmetic mean	S.D.	Arithmetic mean	S.D.	
FACS Lysing Solution	5.36	2.15	1.93	1.74	++ [a:b]
Centrifugation at 200 *g*	42.14	7.12	9.43	3.69	++ [a:b]
Centrifugation at 1000 *g*	49.00	14.70	12.60	4.35	++ [a:b]

+ $P < 0.05$; ++ $P < 0.01$

Proportions of apoptotic neutrophils (%) shown in the table were calculated from the total count of neutrophils. Flow cytometry – apoptotic neutrophils are Annexin-V and propidium iodide positive. Light microscopy - neutrophils with morphological features of apoptosis.

hypotonic lysis. The FACS Lysing Solution method has been commonly used for the isolation of leukocytes for subsequent analysis in FCM (Chen et al. 1996; Bossuyt et al. 1997; Macey et al. 1997; Carulli et al. 1998; Tay et al. 1998; Hodge et al. 1999; Storie et al. 2003). These methods have been chosen because the lysis of erythrocytes is most often used for the isolation of neutrophils. Moreover, one can expect marked effect on the biochemical properties of neutrophils thus isolated, as mentioned below.

The effectiveness of the isolation technique is connected with the cell yield in particular. In this work, FCM established a highly significant difference in neutrophil yield between the method based on hypotonic solution and the method with FACS Lysing Solution, in which the yield was higher. When the light microscope was used, the difference in neutrophil yield between both methods could not be observed, however. This discrepancy was probably caused by the evaluation technique. In the light microscope, "only" 200 neutrophils were evaluated while in the FCM this cell number amounted to twenty thousand.

Two variants of the hypotonic-lysis methods were used, differing in the force *g* used for the centrifugation after the erythrocyte lysis. This was done in order to establish whether the higher centrifugal force (1000 *g*) markedly increased the neutrophil yield and whether it physically altered the cells. This hypothesis was not confirmed, however, as documented by the results obtained in FCM and in the light microscopy. Surprisingly, the use of higher *g* resulted in a lower yield of neutrophils as measured by FCM. This was probably caused by a shift of altered neutrophils from the neutrophil region into the debris region seen in the dot plots.

It has been known that the isolation techniques can induce changes that result in cell death (Van Oostveldt et al. 1999). Apoptosis is the programmed cell death, which is characterised by specific biochemical and structural changes (Kerr and Searle 1973). It is interesting that biochemical changes precede structural changes. One of the techniques used for the detection of apoptosis and necrosis of isolated blood cells in suspension is the FCM after labelling the cells with Annexin-V plus propidium iodide according to Vermes et al. (1995).

During apoptosis, alterations on the cell surface appear first. One of these is the translocation of phosphatidylserine from the inside to the outside of the cytoplasmic membrane. Annexin-V has a high affinity to phosphatidylserine; therefore it is used for the detection of apoptotic cells (Fadok 1992). The translocation of phosphatidylserine to the

cell surface is not specific to apoptosis only, but it also occurs during necrosis. Propidium iodide is a nuclear stain for which the cytoplasmic membrane of both living and apoptotic cells is not permeable, and thus it is suitable for the discrimination between apoptosis and necrosis (Vermes et al. 1995).

Following the translocation of phosphatidylserine in apoptotic cells, further changes appear that can be detected with morphological methods. These changes include karyopyknosis, zeiosis, cell shrinkage and its fragmentation into apoptotic bodies. Despite the fact that the microscopy is not a quick and automated technique, it remains the "gold standard" for the assessment of viability loss of blood cells (Hodge et al. 1999) as it reveals the signs of granulocyte apoptosis in anticoagulated blood (Savill et al. 1993). That is why both above-mentioned detection techniques were used in this work and why this is the first paper in the field of veterinary haematology describing the effects of isolation methods not only on the biochemistry but also on the morphological manifestations of apoptotic neutrophils.

We can thus state that both isolation techniques caused some damage to the neutrophils, manifested as apoptosis and necrosis of some cells. Statistically significant differences were found between both methods of isolation and between the methods of apoptosis detection. The results imply that the method based on the FACS Lysing Solution is a gentler technique of neutrophil isolation compared with the hypotonic lysis. Its effect on the Annexin-V positivity of isolated neutrophils is almost negligible (5%). Considering that normal full blood contains around 2% of Annexin-V positive neutrophils (Van Oostveldt et al. 1999), it is obvious that this technique is not involved in immediate cell alteration. Also, morphological signs of neutrophil apoptosis were observed only in a very small part of the cells (ca 2%). In contrast, when the hypotonic lysis technique was used, the proportion of Annexin-V positive neutrophils and morphologically altered cells observed was higher and the difference was statistically significant.

In accordance with statements of Hodge et al. (1999) the FACS Lysing Solution used in our experiments provides not only complete lysis of erythrocytes, but also fixation of cell membrane of other cells by the fixation compounds including formaldehyde and diethylene glycol in the same time.

Van Oostveldt et al. (1999) have used the FCM method with Annexin-V plus propidium iodide for the detection of apoptosis and necrosis of isolated neutrophils. They have observed 87.3% of Annexin-V positive neutrophils after the isolation by hypotonic erythrocyte lysis. This result is almost identical with that of our experiments with hypotonic erythrocyte lysis and "gentle" centrifugation, which corresponds to the method used by Van Oostveldt et al. (1999). Annexin-V positive neutrophils are apoptotic and necrotic neutrophils, thus this value comprises apoptotic neutrophils (42.14 ± 7.12%) plus necrotic neutrophils (41.12 ± 5.55%). In our study, we have extended the above-mentioned data by the analysis of apoptotic neutrophils with methods of ligh microscopy.

It could seem that there is a discrepancy between the results obtained in the evaluation of the cell population by FCM on the one hand, and the results obtained by light microscopy on the other hand. The results obtained by FCM with Annexin-V plus propidium iodide staining show a higher proportion of apoptotic neutrophils than in the results obtained by light microscopy ($P < 0.01$). However, this fact is substantiated. The dynamics of the apoptotic process include an early stage, characterised by translocation of phosphatidylserine from the outer to the inner side of the cytoplasmic membrane (Vermes et al. 1995). On the other hand, light microscopy detects morphological changes taking place subsequently after the stated biochemical changes of cell receptors (Martin et al. 1995). This implies that both detection methods should be used in the analysis of cellular apoptosis if both the early and the late stages of apoptosis are to be detected.

Because the yield of neutrophils is relatively low, we tried to increase it by using the cell adherence technique besides the blood smears. The adhesion slides showed a markedly higher neutrophil yield in comparison with the smears. The ninety-percent proportion of neutrophils suggests a higher adherence capacity of these cells in comparison with other leukocytes, which confirms the present authors' earlier finding (Ryšánek et al. 2001). Using hypotonic erythrocyte lysis according to Carlson and Kaneko (1973), they attained a yield of 89.9% of neutrophils isolated and adhered to the microscopic slide. The results obtained by FCM and by light microscopy (smears) make it apparent that the yield of neutrophils corresponded to the physiological range of neutrophil proportion in blood leukocyte differential count as reported by Kramer (2000): 15 - 45% of neutrophils in bovine blood.

An interesting finding is the detection of a markedly lower percentage of apoptotic neutrophils ($P < 0.01$) found on the adhesion slides in comparison with that on the smears. This fact is, however, not so much surprising, because it is caused by the different functional properties of apoptotic neutrophils. As it has been known from the literature (Whyte et al. 1993), apoptotic neutrophils have impaired functional properties (cytoskeletal functions, chemotaxis, phagocytosis, degranulation). Greenstein et al. (2000) have reported that decreasing functional activities of neutrophils (including the lower adhesion capacity on epithelial cells) correlates with their apoptosis. Apoptosis of neutrophils is associated with changes at the level of crucial surface receptors, which mediate the adhesion of neutrophils (Dransfield et al. 1995).

In conclusion and on the basis of the results, the methods for the isolation of blood neutrophils can be evaluated with regard to their suitability for subsequent *in vitro* studies: The methods of hypotonic erythrocyte lysis have the disadvantage that the neutrophils (leukocytes) are first isolated and only then the experimental intervention can be performed. Another disadvantage is the demands on time and the need for a large amount of blood, which makes this method inapplicable in small laboratory animals. As documented by the FCM analysis and light microscopy, neutrophils markedly altered by this procedure can load the subsequent studies with a great error. On the contrary, the method with FACS Lysing Solution is very gentle and it allows experimental interventions to be applied first, and only then the neutrophils can be isolated. This leukocyte isolation is very quick and suitable for the purposes of FCM measurements. We must note, however, that no leukocyte isolation method can fully eliminate its effects of neutrophil viability.

Vliv izolačních technik na životnost neutrofilů krve skotu

Byl posouzen vliv vybraných izolačních technik na životnost neutrofilních granulocytů (neutrofilů) krve klinicky zdravých jalovic, kříženek holštýnského a českého strakatého plemene. Byly použity dvě metody izolace krevních neutrofilů: izolace neutrofilů hypotonickou lýzou erytrocytů (ve dvou variantách - dle gravitační síly při odstředění po vlastní lýze erytrocytů: 200 *g* a 1000 *g*) a izolace neutrofilů lytickým činidlem FACS Lysing Solution. Životnost izolovaných neutrofilů byla hodnocena detekcí apoptózy a nekrózy těchto buněk. Z výsledků získaných průtokovým cytometrem (FCM) bylo zjištěno, že z použitých izolačních technik nejméně snižuje životnost neutrofilů metoda s FACS Lysing Solution. Při této metodě bylo z celkového podílu neutrofilů apoptotických 5,36 ± 2,15% a nekrotických 0,51 ± 0,12%. Oproti tomu při izolaci neutrofilů hypotonickou lýzou erytrocytů vykazovala populace neutrofilů výrazně vyšší zastoupení apoptotických a nekrotických buněk (42,14 ± 7,12%, respektive 49,00 ± 14,70% apoptotických neutrofilů a 41,12 ± 5,55%, respektive 36,91 ± 24,38% nekrotických neutrofilů; $P < 0.01$). To rovněž potvrzuje světelná mikroskopie. Z neutrofilů izolovaných pomocí FASC Lysing Solution bylo 1,92 ± 1,74% apoptotických a 1,05 ± 0,76% nekrotických, na rozdíl od hypotonické

lýzy erytrocytů: 9,43 ± 3,69% apoptotických, 12,67 ± 4,74% nekrotických neutrofilů (při odstřeďování s 200 *g*), respektive 12,60 ± 4,35% apoptotických, 14,96 ± 12,64% nekrotických neutrofilů (při odstřeďování s 1000 *g*). Z uvedeného vyplývá, že hypotonická lýza není vhodnou metodou pro izolaci neutrofilů, neboť metoda sama výrazně ovlivňuje životnost buněk.

Acknowledgements

This study was supported by Grant Agency of the Czech Republic (GACR No. 524/03/1531). We thank Mrs Michaela Knakalova for her excellent technical assistance.

References

ARENDS MJ, MORRIS RG, WYLLIE AH 1990: Apoptosis: the role of endonuclease. Amer J Pathol **136**: 593-608

BOSSUYT X, MARTI GE, 1997: Comparative analysis of whole blood lysis methods for flow cytometry. Cytometry **30**: 124-133

BOYUM A 1968: Isolation of mononuclear cells and granulocytes from human peripheral blood. Scand J Clin Lab Invest **21**: 77-89

CARLSON GP, KANEKO JJ 1973: Isolation of leukocytes from bovine peripheral blood. Proc Soc Exp Biol Med **142**: 853-856

CARULLI G, SBRANA S, AZZARA A, MINNUCCI S, ANGIOLINI C, MARINI A, AMBROGI F 1998: Detection of eosinophils in whole blood samples by flow cytometry. Cytometry **34**: 272-279

CHEN WY, LEI HY, WANG JY, LU CC 1996: Direct measurement of neutrophil F-actin content in microvolume whole blood samples. Int Arch Allergy Immunol **110**: 325-331

COWLAND JB, BORREGAARD N 1999: Isolation of neutrophil precursors from bone marrow for biochemical and transcriptional analysis. J Immunol Method **232**: 191-200

DELVECCHIO MT, LEONCINI L, BUERKI K, KRAFT R, MEGHA T, BARBINI P, TOSI P, COTTIER T 1993: Diffuse centrocytic and/or centroblasticmalignant non-Hodgkin's lymphomas: comparison of mitotic and pyknotic (apoptotic) indices. Int J Cancer **124**: 116- 121

DRANSFIELD I, STOCKS SC, HASLETT C 1995: Regulation of cell adhesion molecule expression and function associated with neutrophil apoptosis. Blood **85**: 3264- 3273

DUQUE RE, PHAN SH, HUDSON JL, TILL GO, WARD PA 1985: Functional defects in phagocytic cells following thermal injury. Application of flow cytometric analysis. Amer J Pathol **118**: 116- 127

FADOK VA, VOELKER DR, CAMPBELL PA, COHEN JJ, BRATTON DL, HENSON PM 1992: Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. J Immunol **148**: 2207-2216

GRANVILLE DJ, CARTHY CM, HUNT DW, MCMANUS BM 1998: Apoptosis: molecular aspects of cell death and disease. Lab Invest **78**: 893- 913

GREENSTEIN, S. BARNARD, J, ZHOU, K, FONG, M, HENDEY, B, 2000: Fas activation reduces neutrophil adhesion to endothelial cells. J Leukocyte Biol **68**: 715-722

HALLEN-SANDGREN C, BJORK I 1988: A rapid technique for the isolation of highly purified, functionally intact bovine neutrophilic granulocytes. Vet Immunol Immunop **18**: 81-94

HODGE GL, FLOWER R, HAN P 1999: Optimal storage conditions for preserving granulocyte viability as monitored by Annexin V binding in whole blood. J Immunol Method **225**: 27-38

JUNQUEIRA LC, CARNEIRO J, KELLEY RO 1997: Základy histologie. H a H, Praha, 502 p.

KERR JF, SEARLE J 1973: Deletion of cells by apoptosis during castration-induced involution of the rat prostate. Virchows Arch B Cell Pathol **13**: 87-102

KRAMER JW 2000: Normal haematology of cattle, sheep and goats. In: FELDMAN BF, ZINKL JG, JAIN NC (Eds). Schalm's veterinary haematology. Williams and Wilkins, Philadelphia, pp. 1075-1084.

KUIJPERS TW, ROOS D 2001: Neutrophils. In: Encyklopedia of life sciences. Nature Publishing Group,

MACEY MG, MCCARTHY DA, VAN AGTHOVEN A, NEWLAND AC 1997: How should CD34+ cells be analysed? A study of three classes of antibody and five leucocyte preparation procedures. J Immunol Method **204**: 175-188

MARTIN SJ, REUTELINGSPERGER CP, MCGAHON AJ, RADER JA, VAN SCHIE RC, LAFACE DM, GREEN DR 1995: Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl- 2 and Abl J Exp Med **182**: 1545-1556

MATOUŠKOVÁ O, CHALUPA J, CÍGLER M, HRUŠKA K 1992: STAT Plus - Manual. Veterinary Research Institute, Brno, 168 p.

PAAPE MJ, BANNERMAN DD, ZHAO X, LEE JW 2003: The bovine neutrophil: Structure and function in blood and milk. Vet Res **34**: 597-627

REED JC 2000: Mechanisms of apoptosis. Amer J Pathol **157**: 1415- 1430

ROETS E, BURVENICH C, DIEZ-FRAILE A, NOORDHUIZEN-STASSEN EN 1999: Evaluation of the role of endotoxin and cortisol on modulation of CD18 adhesion receptors in cows with mastitis caused by Escherichia coli. Amer J Vet Res **60**: 2-9

RYŠÁNEK D, BABÁK V, SLÁDEK Z, TOMAN M 2001: Variations among unbred heifers in the activities of polymorphonuclear leucocytes from the mammary gland and blood. J Vet Med B **48**: 31-41

SAVILL JS, FADOK V, HENSON PM, HASLETT C 1993: Phagocytic recognition of cells undergoing apoptosis. Immunol Today **14**: 131-139

SLÁDEK Z, RYŠÁNEK D 2000: Morphology of apoptosis of polymorphonuclear leukocytes isolated from mammary glands of unbred heifers. Vet Med-Czech **45**: 71-81

SMITH DL, ROMMEL F 1977: A rapid micro method for the simultaneous determination of phagocytic-microbiocidal aktivity of human peripheral blood leukocytes in vitro. J Immunol Method **17**: 241-247

SMITH GS 2000: Neutrophils. In: FELDMAN BF, ZINKL JG, JAIN NC (Eds): Schalm's veterinary haematology. Williams and Wilkins, Philadelphia, pp. 281-296.

SOLTYS J, SWAIN SD, SIPES KM, NELSON LK, HANSON AJ, KANTELE JM, JUTILA MA QUINN MT 1999: Isolation of bovine neutrophils with biomagnetic beads: comparison with standard Percoll density gradient isolation methods. J Immunol Method **226**: 71-84

STORIE I, SAWLE A, GOOGFELLOW K, WHITBY L, GRANGER V, REILLY JT, BARNETT D 2003: Flow rate calibration I: a novel approach for performing absolute cell counts. Cytometry B Clin Cytom **55**: 1-7

TAY SP, CHEONG SK, HAMIDAH NH, AINOON O 1998: Flow cytometric analysis of intracellular myeloperoxidase distinguishes lymphocytes, monocytes and granulocytes. Malays J Pathol **20**: 91-94

TROTTER J 2000: WinMDI Version 2.8.

VAN OOSTVELDT K, DOSOGNE H, BURVENICH C, PAAPE MJ, BROCHEZ V, VAN DEN EECKHOUT E 1999: Flow cytometric procedure to detect apoptosis of bovine polymorphonuclear leukocytes in blood. Vet Immunol Immunop **70**: 125-133

VERMES I, HAANEN C, STEFFENS-NAKKEN H, REUTELINGSPERGER C 1995: A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. J Immunol Method **184**: 39-51

WHYTE MK, MEAGHER LC, MACDERMOT J, HASLETT, C 1993: Impairment of function in aging neutrophils is associated with apoptosis. J Immunol **150**: 5124-5134

Zinc Supplementation and Somatic Cell Count in Milk of Dairy Cows

A. PECHOVÁ, L. PAVLATA, E. LOKAJOVÁ

Clinic of Diseases of Ruminants, Faculty of Veterinary Medicine, University of Veterinary and Pharmaceutical Science, Brno, Czech Republic

Received July 27, 2005
Accepted May 11, 2006

Abstract

Pechová A., L. Pavlata, E. Lokajová: Zinc Supplementation and Somatic Cell Count in Milk of Dairy Cows. Acta Vet Brno 2006, 75: 355-361.

The goal of the study was to test the possibility of raising milk zinc (Zn) concentration by increasing the supplementation of Zn, and to assess the effect on the somatic cell count. The experiment was performed at a farm with Czech Pied cattle, with 500 dairy cows and average milk yield 6,390 l. The experiment included 40 dairy cows, assigned to either an experimental (E, n = 20) or a control group (C, n = 20) based on the principle of balanced pairs (efficiency, lactation stage). The experimental group was supplemented Zn in the chelate form (Bioplex Zn, Alltech Inc., Nicholasville, KY), at the dose of 440 mg Zn per animal per day. Blood and milk samples were taken at the start of the experiment and at the end of months 1, 2 and 3.

The average milk Zn concentration during the experiment was 57.36 ± 10.54 $\mu mol \cdot l^{-1}$, ranging from 38.02 to 86.13 $\mu mol \cdot l^{-1}$. A trend towards a positive effect of Zn supplementation on the health of the mammary gland was identified in association with the somatic cell count, which was significantly lower in the experimental group (114.90 ± 68.7·$10^3 \cdot ml^{-1}$ vs. 208.60 ± 148.1·$10^3 \cdot ml^{-1}$; $p < 0.05$) by the end of month 3. A negative correlation was found between the somatic cell count and Zn concentration in milk ($y = -0.0327 x + 61.557$; $r = -0.441$; $p < 0.01$). Our results indicate that milk Zn concentration is not directly affected by the level of zinc supplementation in the feed ration, but zinc supplementation has a positive effect on the somatic cell count.

Cattle, blood, milk, microelements, somatic cell count, organic Zn

Zinc (Zn) is the most abundant intracellular microelement, characterized by a number of catalytic, structural and regulatory functions. Zinc is a biomembrane component playing an essential role in RNA, DNA and ribosome stabilization; it is also present in a number of transcription factors, stabilizes some complexes of hormones and their receptors, plays a role in insulin production and has antioxidant effects. Zn is also crucial for maintenance of integrity and the barrier function of skin and is involved in the immune system in complex ways. All these functions underlie the positive effect of Zn on the health status of the mammary gland (Boland et al. 1996), too. Although the specific mechanism of this action has not been known, the assumption is that the increased resistance of the mammary gland is based on the positive effect of Zn on keratin in the teat duct, its effect on cellular immunity, and the presence of Zn in a number of acute inflammation proteins (Harmon 1998).

The research of zinc has been devoted much of attention in both human and veterinary medicine. To provide a sufficient status of this microelement in both animals and humans, a range of supplements are used, containing mostly inorganic zinc (oxide, sulphate). Organic zinc, which is more readily utilizable, has recently been used, too. As far as human nutrition is concerned, cow milk, containing 2.3 to 6.6 $mg \cdot l^{-1}$ of Zn (Rodriguez et al. 2001), ranks among important sources of zinc. The recommended daily intake of Zn is 12 mg for women and 15 mg for men (Biesalski and Grimm 1999).

Concentration of Zn in cow milk has been a rather underrepresented subject in literature

Address for correspondence:
Doc. MVDr. Alena Pechová, CSc.
Clinic of Diseases of Ruminants
Faculty of Veterinary Medicine
University of Veterinary and Pharmaceutical Sciences Brno
Palackého 1-3, 612 42 Brno
Czech Republic

Phone:+420 541 562 408
Fax:+420 541 562 413
E-mail: pechovaa@vfu.cz
http://www.vfu.cz/acta-vet/actavet.htm

and the factors influencing milk Zn concentration have not been known in detail. In humans, 0.5-1.0 mg of Zn per day is transported through the mammary gland into milk (King 2002) and Zn transportation into milk is hypothesized to be an active process (Kelleher and Lonnerdal 2003). In cow milk Zn primarily binds to casein and, to a small extent, to citrate. Almost 90% of Zn binds to casein in mature milk in contrast to just 60% in the colostrum (Kincaid and Cronrath 1992). In casein, Zn mainly binds to colloid calcium phosphate in casein micelles (Silva et al. 2001).

As far as lactation stages are concerned, colostrum and mature milk differ in terms of Zn content, the concentration of Zn being higher in colostrum than in mature milk. Pavlata et al. (2004) recorded Zn concentration in the first milking colostrum 416.76 ± 120.07 $\mu mol \cdot l^{-1}$. Peak colostrum Zn concentration 12 hours post-parturition published by Vaillancourt and Allen (1991) is 257 ± 14 $\mu mol \cdot l^{-1}$, showing a rapid decrease to 141 ± 8 $\mu mol \cdot l^{-1}$ 24 hours post-parturition and to 82 ± 5 $\mu mol \cdot l^{-1}$ on post-parturition day 3. These authors observed a significant increase in milk Zn concentration after administration of dexamethazone, from which they deduced that the increased Zn levels in colostrum may be due to an increase in glucocorticoids after parturition, resulting in an increased Zn transfer from blood into the mammary
gland.

The goal of the study was to test the possibility of raising milk Zn concentration by increasing the supplementation of this microelement in the chelate form in the feed ration. Additionally, the effect of Zn supplementation on the somatic cell count in milk was studied.

Materials and Methods

The experiment was performed at a farm keeping Czech Pied cattle, whose total capacity was 500 dairy cows. The average milk yield of the herd was 6,390 l. Stanchion housing with bedding was used and the arrangement of the milk on the rows was by their efficiency. They were fed twice daily. A feeding cart was used to distribute total mixed feed ration (1 kg meadow hay, 5 kg grass haylage, 5 kg alfalfa haylage, 21 kg corn silage, 5 kg cossettes, 2 kg grain fodder) computed for the basic milk efficiency of 15 l per day. The cows were additionally supplemented a production grain mixture 0.3 kg per litre of milk. The maximum grain dose was 8 kg per cow at a production yield of 35 l milk. Mineral supplements were administered as part of the basic ration; a small amount was also contained in the production mixture. Zn supplementation was 1,546 mg in the basic feed ration and 14.3 mg per 1 kg in production mixture. Cows fed 8 kg of the mixture thus had 1,660 mg of inorganic Zn per animal per day.

The experiment included a total of 40 dairy cows in the first half of lactation. They were assigned to either an experimental group (E, n = 20) or a control group (C, n = 20) based on the principle of balanced pairs (efficiency, lactation stage). The experimental group was supplemented Zn in the chelate form (Bioplex Zn, Alltech Inc., Nicholasville, KY) at the dose of 2.2 g per animal per day as part of the grain mixture. The minimum guaranteed Zn content in Bioplex Zn was 20%; Zn supplementation was increased by 440 mg for the milk cows in the experimental group. Blood and milk samples were taken at the start of the experiment and at the end of months 1, 2 and 3. The *v. jugularis* was tapped and blood was collected for trace analysis into disposable heparinized plastic tubes. Milk samples were taken from individual milk cows as part of the morning milking and kept free of preservatives; the somatic cell count (SCC) in the samples was determined on the day of collection by a method according to CSN EN ISO 13366-3/1998. Blood plasma and milk Zn concentrations were determined with the help of flame atomic absorption spectrophotometry on an H 1550 device (HILGER, Great Britain). The blood plasma was deproteinized by adding the trichloroacetic acid in the ratio of 1 : 1 for the purposes of the determination proper and Zn content was determined in the supernatant after centrifugation. The milk was mineralized by the wet process with hydrogen dioxide and nitric acid addition (2 ml milk + 1 ml H_2O_2 + 2 ml HNO_3) using the microwave digestion technique in the MLS-1200 (Milestone, Italy) microwave oven. The determination was performed in the laboratory of the Clinic of Diseases of Ruminants of the University of Veterinary and Pharmaceutical Sciences Brno.

The findings were evaluated using F-test to assess the variance of the values for the individual populations and, depending on the result, by the Student *t*-test for populations with equal/unequal variances. SCC was compared between the control and experimental group using the non-parametric Wilcoxon's test. The dynamics of the parameters under analysis was assessed with the help of the matched-pair *t*-test. The results are given as the mean value plus the standard deviation. The individual biochemical indicators under analysis were subjected to correlation and regression analysis. The evaluation was performed using Microsoft EXCEL

software.

Results and Discussion

Milk Zn concentrations varied from 38.02 to 86.13 μmol·l^{-1} throughout the experiment; the mean concentration was 57.36 ± 10.54 μmol·l^{-1}; the medium concentration 57.43 μmol·l^{-1}, and the modal value 61.2 μmol·l^{-1}. The milk Zn concentration found by us in our group of dairy cows was analogical to those quoted by other authors. For a comparison of the milk Zn concentration found by us with some data from literature see Table 1.

Table 1. Comparison of Zn concentration in raw cow milk based on literature data

Zn in milk (μmol·l^{-1})	Reference
57.36 ± 10.54	This work
60.56 ± 2.28	Anderson (1992)
67.43 ± 10.2	Rodriguez et al. (2001)
52.29 ± 0.29	Martin-Hernandez et al. (1992)
56.58 ± 3.36	Zurera et al. (1994)
64.37 ± 6.22	Rincon et al. (1994)
64.38 ± 6.27	Moreno et al. (1993a)
57.80 ± 11.62	Lopez-Mahia et al. (1991)

Although the milk Zn concentrations varied considerably throughout the experiment, no statistically significant differences between the groups were observed in none of the samplings (Table 2). The mean Zn concentration in milk ranged between 40 and 50 μmol·l^{-1} in both the experimental and the control group initially and a significant increase of the Zn

Table 2. Mean concentration of Zn in blood plasma and milk of cows from experimental (E; n = 20) and control groups (C; n = 20) during experiment (mean ± standard deviation)

	Group	Start of the experiment	Month 1	Month 2	Month 3
Zn (plasma) (μmol·l^{-1})	E	12.28 ± 1.01	13.08 ± 1.45	12.70 ± 1.11	12.75 ± 1.02
	C	11.69 ± 1.34	12.16 ± 1.24	12.19 ± 1.18	12.27 ± 0.70
Zn (milk) (μmol·l^{-1})	E	46.05 ± 3.93	64.43 ± 7.12***	60.74 ± 8.00*	57.11 ± 6.69*
	C	47.50 ± 4.03	63.67 ± 9.48***	61.66 ± 7.93	57.73 ± 5.67

* $p < 0.05$ compared to previous sampling
*** $p < 0.001$ compared to previous sampling

level occurred in month 1 of the experimental period. The mean Zn concentration increased by 18 μmol·l^{-1} in the experimental group and by 16 μmol·l^{-1} in the control group. Milk Zn concentration showed no significant change from then until the end of the experimental period. It may be somewhat surprising that an analogical rise in milk Zn concentration occurred in either group after one month of the experimental period despite differing supplementation. We believe that the situation may be due to the incidence of rumen acidosis in the herd before the start of the experiment. The incidence of rumen acidosis was detected by a metabolic test and feed ratios were adjusted prior to the start of the experiment in order to optimize ruminal digestion. The lower milk Zn concentrations thus may have been a reflection of impaired ruminal fermentation prior to the start of the experiment, and a gradual increase of the milk Zn concentration occurred in either group after its improvement. Studies focusing on the relation between milk Zn concentration and the level of supplementation are scarce. Like us, Kirchgessner et al. (1994), who detected only

a non-significant increase in milk Zn content (44.3 μmol·l^{-1} vs. 48.9 μmol·l^{-1}) when supplementing 2.2 g Zn per animal and day in methionin form, did not yield any conclusive proof of an increase in milk Zn concentration either. Strusinska et al. (2004) detected an increase in colostrum Zn concentration by 3 - 10% when supplementing organic microelements. Beňuška et al. (1991) observed an increase in cow milk Zn concentration from 51.5 to 54.4 μmol·l^{-1} on day 8 after intramuscular administration of Zn at 5 mg·kg^{-1} live weight. Other factors influencing cow milk Zn concentration include the breed and the environment. The effect of breed on cow milk Zn concentration was reported by Morales et al. (2000). Holstein cattle showed lower milk Zn concentrations than Jersey cattle (59.6 μmol·l^{-1} vs. 70.3 μmol·l^{-1}). An effect of the environment on milk Zn concentration was observed by Erdogan et al. (2004); higher milk Zn concentrations were reported for a region with no metallurgic industry.

Throughout the experiment, plasma Zn concentrations did not differ significantly between the experimental and the control group either (Table 2). Analogically, no increase in blood Zn concentration was found by Boland et al. (1996), who supplemented 300 mg Zn in bioplex form. A mild increase in Zn concentration occurred in month 2, but was not statistically significant. Throughout the experiment, Zn concentrations were within the lower band of the physiological range 12 - 18 μmol·l^{-1} in both groups (Vrzgula et al. 1990). A more marked drop in Zn concentration below 10 μmol·l^{-1} was detected in 2 cows included in the experimental group only (one at the start of the experiment and second at the end of month 2) and in 8 milk cows included in the control group (4 at the start of the experiment, 2 at the end of month 1 and 2 at the end of month 2). It seems likely that these values indicating a deficiency were not due to insufficient zinc supplementation in the feed rations, but due to reduced absorption of the microelement, e.g. as a consequence of rumen dysfunction or due to some individual anomalies, whose cause is difficult to identify. Blood Zn concentration is modified by other factors such as stress, infection, food intake and hormonal status, too. One of the potential causes may be redistribution of Zn in the organism in association with some pathological processes. Blood serum zinc decreases by 10 - 70% within several hours of the development of the pathological process. Plasma zinc levels decrease to less than 50% during stress, trauma or inflammatory reactions, and do not reflect the real zinc status of the organism (Zadák 2002).

Table 3. Daily milk production and somatic cell count in milk of cows from experimental (E; n = 20) and control groups (C; n = 20) during experiment (mean ± standard deviation)

	Group	Start of the experiment	Month 1	Month 2	Month 3
SCC (10^3·ml^{-1})	E	222.2 ± 71.9	82.1 ± 50.7***	33.5 ± 18.1**	114.9 ± 68.7[a] **
	C	273.5 ± 114.8	80.7 ± 39.7***	55.2 ± 43.7	208.6 ± 148.1[a] **
Daily milk yield(l/day)	E	22.0 ± 5.5	23.4 ± 4.8	22.9 ± 4.2	22.1 ± 4.5
	C	19.9 ± 4.9	21.5 ± 5.4	21.0 ± 4.9	20.3 ± 4.5

[a] $p < 0.05$ comparing the experimental and control group (Wilcoxon's test)
** $p < 0.01$ compared to previous sampling
*** $p < 0.001$ compared to previous sampling

The somatic cell count in milk cows and the mean milk yield per day in either of the groups are presented in Table 3. The experimental and the control group differed significantly at the end of month 3 only, when a lower SCC was recorded in the experimental group (114.9 ± 68.7×10^3·ml^{-1} vs. 208.6 ± 148.1×10^3·ml^{-1}; $p < 0.05$). The somatic cell count however varied considerably throughout the experimental period, with a significant drop of mean values below 100×10^3·ml^{-1} in months 1 and 2. The somatic cell count drop was highly

significant in both groups in month 1 and in the experimental group during month 2, too. A significant increase in SCC was then detected in the last sample-taking, with a more marked increase in the control group, where the mean SCC increased approximately by $150\times10^3\cdot ml^{-1}$ while the increase was only $80\times10^3\cdot ml^{-1}$ in the experimental group. The somatic cell count dynamics testifies to a certain positive effect of increased Zn supplementation, but it seems that it was rather other factors that played the key role.

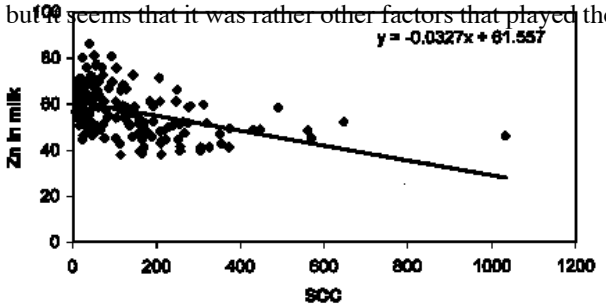

Fig. 1. Relationship between Zn concentration ($\mu mol\cdot l^{-1}$) in milk and somatic cell count ($10^3\cdot ml^{-1}$) in milk of all examined cows (n = 160) with the regression line equation

Milk Zn concentration and the somatic cell count were found to be negatively correlated ($y = -0.0327x + 61.557$; $r = -0.441$; $p < 0.01$) (Fig. 1). Studies evidencing the positive effect of zinc supplementation in the feed rations fed to milk cows on the somatic cell count in milk are available in literature. Harris (1995) published the results of a 90-day trial in whose the experimental group of cows was supplemented Zn in the bioplex form at the dose of 400 mg per animal per day; SCC dropped by 24% in the supplemented group while it increased by 36% in the control group over the same period. Also Andersson et al. (2005) investigated the effect of supplementing complexed zinc (360 mg Zn/day per head) from the beginning of the dry period until 3 months post partum. Cows supplemented with zinc produced numerically lower SCC, but the difference between groups was not significant. Boland et al. (1996) published the results of an experiment in which they supplemented 300 mg Zn in bioplex form, detecting a SCC drop by 45% but no change in blood Zn concentration. An increase in Zn supplementation in the feed rations is not always reflected in an increase in blood Zn concentration, but using our results, the positive effect of Zn supplementation on SCC and/or increased utilization of Zn supplied in the feed ration might be explained by an increased Zn supply into the mammary gland, where it can have a positive effect on the immune function of cells and reduce the number of somatic cells in milk. This increased supply of Zn into the mammary gland can then be reflected by an increase in milk Zn concentration. Only blood Zn concentration was monitored with respect to the incidence of mastitis. Naresh et al. (2001) observed significantly lower blood Zn concentration in cows with subclinical mastitis compared to healthy cows. Middleton et al. (2004) found a decrease in blood serum Zn concentration to 83% in association with *Staphylococcus aureus* experimental infection.

Our results indicate that milk Zn concentration is not directly affected by the level of zinc supplementation in the feed ration, but zinc supplementation has a positive effect on the somatic cell count.

Suplementace zinku u dojnic a počet somatických buněk v mléce

Cílem práce bylo ověření možnosti zvýšení koncentrace zinku v mléce zvýšenou

suplementací tohoto prvku do krmné dávky ve formě chelátu a zhodnocení vlivu zvýšené suplementace zinku na počet somatických buněk v mléce.

Sledování bylo realizováno v chovu českého strakatého skotu o celkové kapacitě 500 ks dojnic a průměrné dojivosti 6 390 l. Do pokusu bylo zařazeno celkem 40 dojnic, které byly na principu analogických dvojic (užitkovost, fáze laktace) rozděleny na skupinu pokusnou (E, n = 20) a kontrolní (C, n = 20). Krmná dávka byla u obou skupin dojnic shodná. Pokusné skupině byl přidáván Zn ve formě chelátu (Bioplex Zn, Alltech Inc. Nicholasville, KY) v dávce 440 mg Zn na kus a den. Na začátku pokusu a dále na konci 1., 2. a 3. měsíce pokusu byly odebírány vzorky krve a mléka. Koncentrace zinku v krevní plazmě a v mléce byla stanovena metodou plamenové atomové absorpční spektrofotometrie.

Suplementace zinku ve formě chelátu do krmné dávky dojnic neovlivnila koncentraci zinku v krevní plazmě ani v mléce. Průměrná koncentrace zinku v mléce v průběhu pokusu byla 57,36 ± 10,54 $\mu mol \cdot l^{-1}$ a pohybovala se od 38,02 do 86,13 $\mu mol \cdot l^{-1}$. Trend pozitivního vlivu suplementace Zn na zdravotní stav mléčné žlázy byl zjištěn v počtu somatických buněk, který byl na konci třetího měsíce signifikantně nižší u pokusné skupiny (114,90 ± 68,7×$10^3 \cdot ml^{-1}$ vs. 208,60 ± 148,1×$10^3 \cdot ml^{-1}$; $p < 0,05$). Mezi počtem somatických buněk a koncentrací zinku v mléce byl zjištěn negativní korelační vztah ($y = -0,0327x + 61,557$; $r = - 0,441$; $p < 0,01$). Na základě našich výsledků není koncentrace Zn v mléce přímo ovlivněna výší suplementace tohoto prvku do krmné dávky, ale zvýšený příjem má pozitivní vliv na počet somatických buněk.

Acknowledgments

This work was supported by MSM Project No. 6215712402 "Veterinary Aspects of Foodstuff Safety and Quality".

References

ANDERSON RR 1992: Comparison of trace elements in milk of 4 species. J Dairy Sci **75**: 3050-3055

ANDERSSON R, LEON L, RAPP CH, SOCHA MT, TOMLINSON DJ 2005: The effect of complexed zinc on somatic cell count and lactate dehydrogenace aktivity in dairy cows. In: Mastitis in dairy production (H. Hogeveen ed.), Wageningen Academic Publisher, The Netherlands, 966 p.

BEŇUŠKA NM, BÍREŠ J, VRZGULA L 1991: Influence of zinc injectable Zindep inj. a.u.v. (Biotika) on zinc content in milk, muscle and liver of ewes, and in cow milk. Biopharm J Vet Pharm **1**: 111-114

BIESALSKI HK, GRIMM P 1999: Taschenatlas der Ernahrung. Georg Thieme Verlag Stuttgart, 314 p.

BOLAND MP, O'DONNELL GO, O'CALLAGHAN D 1996: The contribution of mineral proteinates to production and reproduction in dairy cattle. In: Biotechnology in the Feed Industry, Proceedings of the 12th Annual Symposium (T.P. Lyons and K. A. Jacques, eds.). Nottigham University Press, Loughborough, Leics, UK, pp. 95-102

ERDOGAN S, CELIK S, ERDOGAN Z 2004: Sesonal and locational effects on serum, milk, liver and kidney chromium, manganese, copper, zinc, and iron concentrations of dairy cows. Biol Trace Elem Res **98**: 51-61

HARMON RJ 1998: Trace minerals and dairy cattle: importance for udder health. In: Biotechnology in the Feed Industry, Proceedings of the 14th Annual Symposium (T.P.Lyons and K. A. Jacques, eds.). Nottigham University Press, Loughborough, Leics, UK, pp. 485-496

HARRIS B 1995: The effect of feeding zinc proteinate to lactating dairy cows. In: Biotechnology in the Feed Industry, Proceedings of the 11th Annual Symposium (T.P.Lyons and K. A. Jacques, eds.). Nottigham University Press, Loughborough, Leics, UK, pp. 299-306

KELLEHER SL, LONNERDAL B 2003: Zn transporter levels and localization change throughout lactation in rat mammary gland and are regulated by Zn in mammary cells. J Nutr **133**: 3378-3385

KINCAID RL, CRONRATH JD 1992: Zinc concentration and distribution in mammary secretions of prepartum cows. J Dairy Sci **75**: 481- 848

KING JC 2002: Enhanced zinc utilization during lactation may reduce maternal and infant zinc depletion. Am J Clin Nutr **75**: 2-3

KIRHGESSNER M, PAULICKS BR, HAGEMEISTER H 1994: Zinc concentration in the milk of dairy cows supplemented with high-levels of zinc methionine. J Anim Physiol An N **72**: 165-167

LOPEZ-MAHIA, P, PASEIRO-LOSADA, P, SIMAL-LOZANO, J 1991: Elementos traza en leche natural de vaca. Aliment **226**: 45-47

MARTIN-HERNANDEZ C, AMIGO L, MARTIN-ALVAREZ PJ, JUAREZ M 1992: Differentiation of milks and cheeses according to species based on the mineral content. Z Lebensm Unters For **194**: 541-544

MIDDLETON JR, LUBY CD, VIERA L, TYLER JW, CASTEEL S 2004: Short communication: Influence of Staphylococcus aureus intramammary infection on serum copper, zinc, and iron concentrations. J Dairy Sci **87**: 976-979

MORALES MS, PALMQUIST DL, WEISS WP 2000: Milk fat composition of Holstein and Jersey cows with control or depleted copper status and fed whole soybeans or tallow. J Dairy Sci **83**: 2112- 2119

MORENO R, AMARO M, ZURERA G 1993: Micronutrients an natural cow, ewe and goat milk. Int J Food Sci Nutr **44**: 37-46

NARESH R, DWIVEDI SK, DEY S, SWARUP D 2001: Zinc, copper and cobalt concentrations in blood during inflammation of the mammary gland in dairy cows. Asian Austral J Anim Sci **14**: 564- 566

PAVLATA L, PECHOVÁ A, DVOŘÁK R 2004: Microelements in Colostrum and Blood of Cows and their Calves during Colostral Nutrition. Acta Vet Brno **73**: 421-429

RINCON F, MORENO R, ZURERA G, AMARO M 1994: Mineral composition as a characteristic for the identification of animal origin of milk. J Dairy Res **61**: 151-154

RODRIGUEZ EMR, ALAEJOS MS, ROMERO CD 2001: Mineral concentrations in cow's milk from the Canary Island. J Food Comp Anal **14**: 419-430

SILVA FV, LOPES GS, NOBREGA JA, SOUZA GB, NOGUEIRA ARA 2001: Study of the protein-bound fraction of calcium, iron, magnesium and zinc in bovine milk. Spectrochim Acta B **56**: 1909-1916

STRUSINSKA D, MIEREJEWSKA J, SKOK A 2004: Concentration of mineral components, beta-carotene, vitamins A and E in cow colostrum and milk when using mineral-vitamin supplements. Med Weter **60** : 202-206

VAILLANCOURT SJ, ALLEN JC 1991: Glucocorticoid effects on zinc transport into colostrum and milk of lactating cows. Biol Trace Elem Res **60**: 185-196

VRZGULA et al. 1990: Poruch látkového metabolismu hospdárskych zvierat a ich prevencia. Bratislava, Príroda, 2nd edition, 503 p.

ZADÁK Z 2002: Výživa v intenzivní péči. Grada Publishing a.s., 496 p.

ZURERA G, MORENO R, AMARO M 1994: Effect of processing on contents and relationships of mineral elements of milk. Food Chem **51** : 75-78

ACTA VET. BRNO 2006, 75: 363-372; doi:10.2754/avb200675030363

Effect of Lupine and Amaranth on Growth Efficiency, Health, and Carcass Characteristics and Meat Quality of Market Pigs

Z. ZRALÝ[1], B. PÍSAŘÍKOVÁ[1], M. TRČKOVÁ[1], I. HERZIG[1], M. JŮZL[2], J. SIMEONOVOVÁ[2]

[1]Veterinary Research Institute, Brno, Czech Republic
[2]Mendel University of Agriculture and Forestry, Czech Republic

Received October 27, 2005
Accepted June 30, 2006

Abstract

Zralý Z., B. Písaříková, M. Trčková, I. Herzig, M. Jůzl, J. Simeonovová: Effect of Lupine and Amaranth on Growth Efficiency, Health, and Carcass Characteristics and Meat Quality of Market Pigs. Acta Vet Brno 2006, 75: 363-372.

The purpose of the present study was to ascertain whether it is possible to substitute animal protein in a pig diet with lupine of Sonet cultivar or amaranth grain or dried surface biomass of amaranth when one type feed ration is used during the entire period of fattening, and to investigate its impact on growth efficiency and health of pigs. Its role in feed conversion, carcass characteristics, meat quality and sensory parameters was analyzed.

Four groups of pigs (n = 10, 5 males and 5 females) with the body weight of 24 kg were fed *semi-ad libitum* for 90 days the following diets: control group (C) - diet containing 3% of fish meal, experimental group (1) - diet containing 5% of non-heat-treated amaranth grain and 5% of dried surface biomass of amaranth, experimental group (2) - diet containing 5% of popped (heat-treated) amaranth grain and 5% of dried surface amaranth biomass, experimental group (3) - diet containing 10% of lupine seed meal. Animal protein substitution in diets with amaranth (group 1, 2) or lupine (3) did not result in significant differences ($p > 0.05$) in average daily body weight gain in comparison with the control group (C - 0.83 and 0.82, 0.80 and 0.79 kg in groups 1, 2 and 3, respectively). The differences in feed conversion were non-significant (2.69 to 2.79 kg/kg of body weight gain). The tested diets did not adversely affect animal metabolism, and significantly lower concentrations ($p < 0.05$, $p < 0.01$) of total protein, glucose and triacylglycerol in animals of group C ranged within physiological limits. No significant differences between control and experimental animals were found in carcass characteristics, meat and sensory qualities. Lupine supplementation of the diet positively affected the tenderness and taste of meat.

Animal protein, performance, feed conversion, blood biochemistry, intramuscular fat, sensory parameters

After the limitation of animal-derived feeds in animal nutrition, concern has been expressed as to what substances to use to replace the high-quality raw material previously used for the preparation of combined feeds without adverse effects on the health status and performance of fattened pigs. Although it is very difficult to replace animal protein, leguminous plants (soy, pea and lupine), oil plants (oilseed rape and sunflower) and pseudocereals (amaranth) may be considered. Predominant use of imported soy as a feed component for monogastric animals has been limited by price and moreover, heat treatment is necessary for inactivation of anti-nutrient substances. Some cultivars of soy also contain oestrogen-like substances (Sommer 2003).

It follows from the present situation that it is necessary to use vegetable feeds of inland production and of high nutritional quality that are available for a reasonable price. The seeds of lupine cultivars meet these requirements from an aspect of high protein content, adaptability to various climatic conditions and sufficient yield. According to the most recent studies, lupine has been classified as one of eight prospective protein sources for the production of feeds and foods that may replace meat in human nutrition (Dijkstra et al. 2003).

Address for correspondence:
MVDr. Zdeněk Zralý, CSc.
Veterinary Research Institute
Hudcova 70
621 32 Brno, Czech Republic

Phone: +420 533 331 612
Fax: + 420 541 211 229
E-mail: zraly@vri.cz
http://www.vfu.cz/acta-vet/actavet.htm

The seeds of sweet lupine cultivars (*Lupinus albus, L. angustifolius, L. luteus*) contain 28 to 48% N-substances in dry matter, which depends on the lupine cultivar and climatic conditions (Hove 1974; Green and Oram 1983; Sousa et al. 1996; Linnemann and Dijkstra 2002). The profile of amino acids is characterized by a lower level of sulphur containing amino acids and threonine in comparison with soy (Simon and Jeroch 1999); in contrast, arginine content is markedly higher (Suchý et al. 2005). The lipid content is 5 to 13%, high percentages (up to 80%) of unsaturated fatty acids are represented by linoleic and linolenic acids (Yanez et al. 1983) and average levels of metabolized energy for pigs are slightly lower in comparison with soy. In contrast to other leguminous plants, the lupine seed contains more crude fibre; a proportion of that is viewed as dietetically beneficial. The seed contains minute amounts of starch (5 to 12%), higher levels of soluble non-starch polysaccharides (NSP) and alpha-galactosides that cannot be digested by endogenous enzymes; under such conditions, decreased utilization of nutrients and energy, disturbed health status and low performance of pigs have been recorded (Batterham 1992; Gdala et al. 1997 et al.). The content of anti-nutrient substances, particularly quinolizidine alkaloids, markedly decreased in new sweet lupine cultivars in comparison with bitter cultivars (Aniszewski et al. 2001). The content of other anti-nutrient substances (trypsin and chymotrypsin inhibitors, tanins, phenolic substances, lectins etc.) is relatively low and the seeds of these cultivars do not require heat treatment and may be fed unprocessed to the animals.

It was confirmed in animal experiments that amaranth contains high-quality protein. However, in some cases, the variables of growth efficiency obtained were lower than expected (Correa et al. 1986; Imeri et al. 1987). The possibility to use autoclaved grain for chickens was tested by Acar et al. (1988), the effects of extruded amaranth grain in feed rations were investigated by Tillman and Waldroup (1987) and nutritional quality of the surface biomass was studied in lambs by Pond and Lehmann (1989). However, available literature data on the amaranth use in pig nutrition are scarce (Sokół et al. 2001; Zralý et al. 2004).

The purpose of the present study was to ascertain whether it is possible to substitute animal protein in a pig diet with lupine of the Sonet cultivar and amaranth, using one type feed ration during the entire period of fattening, and its impact on the pigs' growth efficiency and health, carcass characteristics and meat quality.

Materials and Methods

Forty Large White x Landrace pigs in equal numbers of barrows and gilts (mean live weight 24.4 ± 1.70 kg) were used. The animals were marked by tattooing. Experimental animals were housed in pens of 10 pigs each, under good hygienic conditions of accredited animal facilities at the Veterinary Research Institute. Average surface space was 1.7 m^2 and the length of the feeding place was 0.3 m per pig. Straw was used as bedding.

Conditions of animal hygiene were monitored by continuous measurements of ambient temperature and relative humidity. Average morning and evening ambient temperatures in pig stables were 20.8 ± 2.4 °C and 22.4 ± 2.3 °C, respectively; average relative humidity was 53.5 ± 5.9% and 57.1 ± 6.6%, respectively.

The experiment was preceded by an 11-day pre-treatment period, during which the pigs gradually adapted to the new diet and environment, and concurrently their performance and growth uniformity were monitored. They were preventively dewormed (Ivomec, inj.; MSD, Agvet, USA) during that period with doses according to the manufacturer's instructions.

Before biological testing commenced, the animals were weighed, and based on individual live weight, they were allocated to four groups (5 males and 5 females in each). Average weight of pigs in respective groups ranged between 24.2 ± 1.4 and 24.8 ± 1.8 kg. Variation coefficient (< 10%) was consistent with the requirements of biological testing (Anonymous 2004).

Experimental diets were based on cereals (wheat and barley) and comprised extracted soy meal, 46% crude protein (CP) and feed supplements. Control group diet (C) contained 3% of fish meal. The diets for three experimental groups contained 5% of amaranth flour and 5% of dried green surface amaranth biomass (1), 5% of popped (heat-treated) amaranth grain and 5% of dried green surface amaranth biomass (2) or 10% of dehulled ground lupine seed of theSonet cultivar (3). Compositions of experimental diets are given in Table 1. The diets were

suggested for fattening of meat type pigs with 56% proportion of lean musculature (Šimeček et al. 1993). The diets were analysed for the content of basic nutrients using laboratory procedures for testing the feeds (Anonymous 2000).

The diets were mixed with drinking water 1 : 1 and fed to each group of pigs twice a day during the experimental period (90 days), partially *ad libitum*. Water was available *ad libitum*.

The amounts of feed were regulated according to the current intake and the weight of the animals in order to reduce food refusals. Thirty minutes after the beginning of feeding, the refusals were removed, weighed and taken into account in subsequent calculations.

Individually identified pigs were weighed at the beginning of the trial and then at one-week intervals. Individual and group body weight gains and relative growth rates were calculated from the detected live body weight of the animals according to Karakoz (1986). Feed conversion was calculated from feed consumption and the body weight gains of respective groups of animals.

At the beginning and the end of the trial, blood samples were drawn from *v. cava cranialis* for biochemical analysis. Total protein (TP), albumin (Alb), glucose (Glu), triacylglycerols (TG), cholesterol (Chol), HDL and LDL lipoproteins, alkaline phosphatase (ALP), aspartate and alanine aminotransferases (AST, ALT), calcium (Ca), and phosphorus (P) blood plasma levels were determined using Bio-La-Tests (PLIVA - Lachema Brno Ltd., Czech Republic). The health status of animals was monitored daily by observation at regular intervals. Occasional morbidity and mortality were recorded.

All experimental animals were slaughtered in an experimental slaughterhouse using electrical stunning and exsanguination. Carcass quality of slaughtered animals was assessed using the following criteria: dressing percentage, estimated lean yield in percent (ZP method, ČSN 46 61 60), backfat thickness and *musculus longissimus lumborum et thoracis* (MLLT) depth were measured with a slide gauge at the location of the last thoracic vertebra. At the same location of MLLT, pH values 1 (pH_1) and 24 hours (pH_{24}) after slaughter were measured using WTW 720 pH meter (Inolab, BRD). Dry matter, crude protein (N x 6.25) and fat content (petrolether extraction in Soxhlet's extraction equipment for 6 h) were determined in MLLT.

The samples (app. 500 g) of muscle tissue were collected from MLLT for analyses of sensory parameters. Evaluation was done by a group of 8 members meeting the requirements of ISO 8586-1 in a special room for analysis of sensory parameters (according to ISO 8589). A five-point scale was used (1 - the least desirable, 5 - the most desirable). The following attributes (descriptors) of meat were assessed: colour, texture, juiciness, odour and taste.

The results obtained were processed by statistical methods using statistical and graphic software STAT Plus (Matoušková et al. 1992). Basic statistical data such as the arithmetic average (x) and standard deviation (SD) were calculated. Student's *t*-test was used for the evaluation of significance of differences between the detected averages (* $p < 0.05$, ** $p < 0.01$).

Results and Discussion

The investigation of the respective diet effects on selected performance and health characteristics was conducted under objective conditions. The contents of N-substances and MEp ranged between 170.7 and 174.2 g/kg, and 13.1 and 13.3 MJ/kg, respectively. The lysine/MEp ratio was identical for all the diets (0.83 g/MJ). Table 1 shows the content of other nutrients. Both the control and experimental diets were isoproteinic and isoenergetic.

The Sonet lupine analysis performed in this study showed that the content of N-substances was 360.0 g/kg in 100% of dry matter; which increased by 24.4% (448.1 g/kg) after the dehulling of the lupine seed. These values are in concord with the nutrient contents published by Suchý et al. (2005). The nutrient profile of lupine is similar to that of extruded ground soy (Zeman et al. 1995), and its nutritional value for monogastric animals is high.

The live body weight of control and experimental animals at the beginning of the experiment was steady and variation coefficients were adequate (v = 5.8 to 7.6%). The live body weight and average body weight gains on days 30, 60 and 90 of experiment are presented in detail in Table 2. The total body weight gain was 74.6 kg in the control group and 73.5, 72.1 and 71.4 kg in experimental groups 1 to 3, respectively. Differences between groups were non-significant. The following average daily weight gains in these groups were: 0.83, 0.82, 0.80 and 0.79 kg, respectively, and relative growth rates were: 300.8, 302.4, 297.9 and 294.0%, respectively. The results of the present study corresponded to the results obtained by Sokól et al. (2001) who included 25% of amaranth flour or popped amaranth grain into the diets, and to the results from our previous study (Zralý et al. 2004). The highest growth intensity was recorded between days 31 and 60 of the experiment. Significant

Table 1. Composition and nutrient contents in the diets

Components (%)	Diet[1]			
	C	**1**	**2**	**3**
Barley	40.00	30.00	30.00	30.00
Wheat	43.00	42.70	42.90	48.60
Extracted soy meal 46% CP	11.00	14.30	14.20	8.20
Fish meal 64% CP	3.00	-	-	-
Amaranth DAB[2]	-	5.00	5.00	-
Amaranth GAG[2]	-	5.00	-	-
Amaranth PAG[2]	-	-	5.00	-
Lupine	-	-	-	10.00
Lysine 60%	0.12	0.06	0.06	0.18
Methionine 40%	-	-	-	0.05
Threonine 20%	0.10	0.05	0.05	0.10
Ground limestone	-	-	-	0.10
Unimak P1-M[3]	2.80	2.80	2.80	2.80
Nutrients (g/kg)				
Dry matter	893.2	899.8	893.8	883.7
Nitrogenous substances	171.2	174.2	170.7	170.0
Fat	19.1	19.2	19.8	22.6
Fibre	31.7	40.4	38.2	27.9
Ash	53.9	51.0	47.8	42.8
Total	275.9	284.8	276.5	266.2
N-FE[4]	617.3	615.0	617.3	621.5
OM[5]	839.3	848.8	846.0	840.9
MEp (MJ/kg)[6]	13.2	13.1	13.1	13.3
Lysine /MEp (g./MJ)	0.83	0.83	0.83	0.83

[1] C – control diet 1 – DAB + GAG 2 – DAB + PAG 3 – lupine
[2] DAB – dried surface amaranth biomass GAG – ground amaranth grain PAG – popped amaranth grain
[3] Commercial supplement contained the following per kg: 335 000 IU vitamin A, 45 000 IU vitamin D, 125 mg vitamin K, 2 665 mg vitamin E, 5.3 mg vitamin B^1, 165 mg vitamin B^2, 14 mg vitanmin B^6, 1.10 mg vitamin B^{12}, 165 mg niacin, 250 mg pant. calcium, 1000 mg cholinchlorid, 0.8 mg biotin, 6 600 mg vitamin C, 110 g L-lysine HCl, 33 g D,L-methionine, 55 g L-threonine, 15 mg Co, 65 mg J, 11 mg Se, 660 mg Cu, 1 585 mg Mn, 3 500 mg Zn, 2 080 mg Fe, 56 g Na, 12 g Mg, 80 g P, 205 g Ca, 833 mg Endox, 11 250 mg Bio-plus 2B, 2 900 mg Natuphos 5 000G, 665 mg Sacharin
[4] N-FE- nitrogen-free extracts [5] OM-organic matter [6] MEp-metabolizable energy

differences ($p < 0.05$) were found between experimental groups 1 and 2 (0.97 ± 0.06 and 0.89 ± 0.05 kg/head/day). The average body weight gains in the control group and group 3 were lower by 3.5 and 4.2%, respectively (0.94 ± 0.07 and 0.93 ± 0.05 kg/head/day, respectively). Lower performance of experimental group 2 fed a diet containing 5% of popped amaranth grain can be explained by a decreased digestibility of nutrients after heat treatment. Due to the technological treatment, essential amino acids may degrade and consequently their content may be decreased or changed into a racemic compound; non-digestible complexes of fibre components with proteins or amino acids may also be formed (Bressani et al. 1987; Tovar et al. 1989).

The average consumption of the diet per head and day was 2.26 kg in the control group and 2.20, 2.22 and 2.21 kg in the experimental groups 1 to 3, respectively. The control diet conversion was 2.72 kg per kg of body weight gain and the conversion of experimental diets was 2.69, 2.77 and 2.79 kg, respectively. A slightly lower performance of the experimental group 3 fed the diet containing 10% of lupine might be explained after analysis of essential

Table 2. Growth performance of pigs fed different diets (kg)

	Live weight	Live weight	Weight gain	Live weight	Weight gain	Weight gain	Live weight	Weight gain	Weight gain
Diet[1]	Day 0	Day 30	0 - 30	Day 60	31 - 60	0 - 60	Day 90	61 - 90	0 - 90
C									
Mean	24.8	45.5	20.7	73.9	28.4	49.1	99.4	25.5	74.6
± SD	1.80	3.87	2.52	5.90	3.71	4.76	6.06	4.28	4.74
Index %	100	100	100	100	100	100	100	100	100
1									
Mean	24.3	43.1	18.8	72.3	29.2[a*]	48.0	97.8	25.5	73.5
± SD	1.83	4.95	3.60	6.22	2.15	4.97	8.02	4.58	7.21
Index %	98.0	94.7	90.0	97.8	102.8	97.8	98.4	100	98.5
2									
Mean	24.2	44.6	20.4	71.4	26.8	47.2	96.3	24.9	72.1
± SD	1.42	2.07	1.90	4.09	2.70	3.86	5.87	3.11	6.05
Index %	97.6	98.0	97.6	96.6	94.4	96.3	96.9	97.6	96.6
3									
Mean	24.3	43.3	19.0	71.3	28.0	47.0	95.7	24.4	71.4
± SD	1.64	4.90	3.55	7.80	3.68	6.51	9.72	4.22	8.69
Index %	98.0	95.2	90.9	96.5	98.6	95.7	96.3	95.7	95.7

[1] C – control diet 1 – DAB + GAG 2 – DAB + PAG 3 – lupine

[a*] $p < 0.05$ significant difference between diet 1 and 2

amino acids, particularly limiting methionine, lysine and threonine (Batterham 1992; Gdala et al. 1997).

The health status of animals was investigated both clinically and biochemically by the analysis of blood plasma. At the beginning of the experiments, selected biochemical indices ranged within physiological values and gave evidence of a good homogeneity of the experimental groups. The effect of the diets on blood variables is presented in Table 3. Non-significant differences in albumin, cholesterol and inorganic phosphorus levels, AST and ALP activities were detected. The TP content was significantly decreased in the control group in comparison with groups 1 and 3 ($p < 0.05$ and $p < 0.01$, respectively); Glu was likewise decreased in comparison with group 1 ($p < 0.05$). Highly significant differences were found for TG levels in control group in comparison with groups 1 and 3 ($p < 0.01$) and group 2 ($p < 0.05$; 0.22 and 0.30 mmol/l, respectively); HDL level in group C was also significantly decreased in comparison with group 2 ($p < 0.05$; 0.56 and 0.64 mmol/l, respectively). The AST activity detected in all the groups of animals was below the upper reference limit given by Tlučhoř (2001). Calcium concentration was at the upper limit of physiological range in all the groups. It follows from the blood plasma TG levels that no hypolipidemic effect of amaranth was recorded in contrast to Grajeta (1999). No clinical signs of a disease were observed and no mortality or gross changes of internal organs were recorded during the entire experiment.

The effect of the diets on selected characteristics of pig carcasses was assessed after the experiment had been completed. No significant between-group differences in dressing percentages and estimated lean yields ($p > 0.05$) were detected. The obtained values corresponded to the data described by Ševčíková et al. (2002). Backfat thickness and

Table 3. Selected biochemical characteristics of blood plasma

Parameters[2]	Sample	Diet[1]							
		C		**1**		**2**		**3**	
		x	± SD	x	± SD	x	± SD	x	± SD
TP (g/l)	1st	53.5	4.63	54.3	4.17	57.4	3.72	58.4	5.73
	2nd	54.0 ^{C,a}	3.20	56.8 ^{a}	2.68	57.7	4.88	58.8 ^{C}	3.33
Alb (g/l)	1st	23.2	2.86	21.4	0.97	21.6	2.01	21.4	1.43
	2nd	30.8	3.33	32.7	1.49	32.4	1.90	31.3	2.33
Glu (mmol/l)	1st	5.80 ^{c}	0.85	5.48	0.65	5.37	0.58	4.83 ^{c}	1.10
	2nd	4.10 ^{a}	0.39	4.67 ^{a}	0.68	4.44	0.52	4.35	0.30
TG (mmol/l)	1st	0.30 ^{B,c}	0.08	0.39	0.15	0.39 ^{B}	0.04	0.41 ^{c}	0.13
	2nd	0.22 ^{A,C,b}	0.04	0.33 ^{A}	0.08	0.30 ^{b}	0.08	0.29 ^{C}	0.05
Chol (mmol/l)	1st	2.14	0.29	1.90	0.40	2.17	0.43	1.98	0.54
	2nd	1.78	0.27	1.81	0.24	1.78	0.23	1.85	0.35
HDL (mmol/l)	1st	0.62	0.13	0.56	0.11	0.60	0.08	0.60	0.12
	2nd	0.56 ^{b}	0.08	0.64	0.12	0.64 ^{b}	0.09	0.67	0.14
LDL (mmol/l)	1st	1.12	0.19	0.98	0.16	1.07	0.13	1.09	0.21
	2nd	0.97	0.14	0.99	0.13	0.95	0.13	0.99	0.12
ALT (µkat/l)	1st	0.35	0.07	0.33	0.04	0.31	0.05	0.35	0.07
	2nd	0.45 ^{a,b}	0.05	0.39 ^{a}	0.05	0.37 ^{b}	0.09	0.39	0.09
AST (µkat/l)	1st	0.35	0.09	0.35	0.07	0.35	0.08	0.40	0.09
	2nd	0.18	0.12	0.20	0.08	0.17	0.12	0.30	0.18
ALP (µkat/l)	1st	1.75	0.38	1.78	0.43	1.70	0.34	1.64	0.44
	2nd	1.83	0.41	1.76	0.45	1.52	0.26	1.62	0.29
Ca (mmol/l)	1st	2.32	0.33	2.33	0.63	2.43	0.29	2.28	0.20
	2nd	3.03 ^{c}	0.24	3.09 ^{e}	0.27	2.99	0.36	2.70 ^{c,e}	0.30
P (mmol/l)	1st	2.56	0.24	2.70	0.27	2.53	0.22	2.76	0 25
	2nd	2.42	0.12	2.43	0.16	2.42	0 13	2.39	0 19

[1] C – control diet 1 – DAB + GAG 2 – DAB + PAG 3 – lupine

[2] TP – total proteins Alb – albumin Glu – glucose TG – triacylglycerols Chol – cholesterol HDL – HDL lipoproteins LDL – LDL lipoproteins ALT – alanine transaminase AST – aspartate transaminase ALP – alkaline phosphatase

[a-e] – significant difference ($p < 0.05$) between values with the same letters in a row

[A-C] – significant difference ($p < 0.01$) between values with the same letters in a row

MLLT depth in the control group were almost identical with groups of animals fed amaranth or lupine containing diets (Table 4). The above-mentioned carcass characteristics corresponded to the findings of Cannon et al. (1992) and Leikus et al. (2002) who did not find any differences when using various diets supplemented with soy, field beans and sweet lupines. The average pH_1 and pH_{24} values ranged between 6.38 and 6.51, and 5.32 and 5.42, respectively. The between-group differences were non-significant. The data obtained in the present study ranged within values indicating standard meat quality and were in agreement with Oliver et al. (1993) and Ševčíková et al. (2002).

The results of physical and chemical analyses of MLLT (dry matter, crude protein) did not show any significant effect of experimental diets on meat composition, and corresponded to the findings of various authors (Nadějе et al. 2000; Ševčíková et al. 2002). The content of intramuscular fat ranged between 1.84 and 2.22% and approached the requirement for optimum sensory parameters of pork defined by Fernandez et al. (1999). The intramuscular fat content was significantly lower ($p < 0.05$) in experimental group 2 in comparison with animals fed the lupine containing diet (group 3).

Table 4. Carcass characteristics of pigs fed different diets

Indices	Diet[1]			
	C	**1**	**2**	**3**
BW[2] before slaughter (kg)	100.6 ± 5.9	99.1 ± 6.9	97.5 ± 3.8	97.1 ± 8.0
Dressing percentage (%)	79.4 ± 0.4	79.2 ± 0.1	77.8 ± 0.2	79.9 ± 0.3
Estimated lean yield (%)	58.6 ± 2.5	58.0 ± 2.3	58.8 ± 1.9	59.3 ± 2.1
Backfat thickness (mm)	23.7 ± 1.7	23.6 ± 1.9	24.0 ± 1.5	22.9 ± 1.7
MLLT depth (mm)	57.8 ± 1.3	57.5 ± 1.8	58.2 ± 1.2	58.9 ± 1.9
pH_1	6.38 ± 0.1	6.41 ± 0.1	6.44 ± 0.1	6.51 ± 0.2
pH_{24}	5.32 ± 0.1	5.37 ± 0.1	5.33 ± 0.1	5.42 ± 0.2
Contents in *m. longissimus dorsi*				
Dry matter (%)	25.76 ± 0.3	25.75 ± 0.4	25.34 ± 0.4	25.67 ± 0.4
Crude protein (%)	22.22 ± 0.5	22.04 ± 0.4	22.00 ± 0.6	21.97 ± 0.4
Intramuscular fat (%)	2.05 ± 0.3	2.22 ± 0.2 [a]	1.84 ± 0.3 [b]	2.20 ± 0.1 [a]
Sensory evaluation				
Colour	4.03 ± 0.67	3.65 ± 0.78	3.59 ± 0.87	3.66 ± 0.95
Texture	3.69 ± 0.80	3.64 ± 0.86	3.55 ± 1.05	3.85 ± 0.87
Juiciness	3.51 ± 0.86	3.60 ± 0.77	3.50 ± 1.03	3.51 ± 0.98
Odour	4.21 ± 0.74	4.05 ± 0.65	3.90 ± 0.63	4.09 ± 0.80
Taste	3.68 ± 0.76	3.76 ± 0.72	3.58 ± 0.91	3.81 ± 0.84

[1] C – control diet 1 – DAB + GAG 2 – DAB + PAG 3 – lupine
[2] body weight
[a, b] – $p < 0.05$

No statistically significant differences ($p > 0.05$) were obtained by the analysis of all five selected sensory parameters from the viewpoint of sex of the animals (Table 4). Colour, texture and odour of meat from gilts more approached the requirements than meat from barrows. Meat from barrows was slightly more juicy and palatable than meat from gilts. Comparison of the results of sensory analysis from an aspect of the effect of the used diets without respect to sex, no significant differences ($p > 0.05$) were found in any of the investigated descriptors (Table 4). The lightest colour of meat and the best odour was found in the control group fed a diet supplemented with animal protein (fish meal). The juiciest

meat was obtained from animals of experimental group 1 given a diet containing non-heat treated amaranth grain. Meat from experimental animals of group 4 fed a diet supplemented with 10% of dehulled lupine showed to be the most tender (most demanded) and most palatable. References concerning the effect of tested raw materials used for feeding, on sensory parameters of meat are scarce. Sokól et al. (2001) investigated the effect of amaranth on sensory parameters of meat; they did not find any difference in meat from pigs fattened in a standard way and pigs fed diets supplemented with 25% of amaranth. Leikus et al. (2004) detected reduced meat colour in animals fed diets containing 15 and 20% of white lupine, cultivar Danko, in comparison with animals fed diets containing 18% of extruded soybeans or 20 and 25% of field beans. It follows from the present study that no adverse effects from the aspect of consumer's demands for meat sensory parameters were detected in any of the tested diets. A beneficial effect of lupine on tenderness and taste of meat was shown.

It follows from the data obtained by evaluation of growth performance of pigs, feed conversion, health status, carcass and meat quality, including sensory parameters that animal protein may be replaced with vegetable protein, in this case with lupine and amaranth. Due to a decreased effectiveness of the lupine containing diet, more detailed analysis of amino acid profile from the aspect of limiting amino acids will be necessary.

Vliv lupiny a amarantu na užitkovost, zdraví, jatečnou hodnotu a kvalitu masa výkrmových prasat

Cílem práce bylo ověřit možnost náhrady živočišné bílkoviny v dietě prasat lupinou, var. Sonet nebo amarantem ve formě zrna nebo sušené nadzemní biomasy s použitím stejné krmné směsi po celé období výkrmu a vliv na růstové schopnosti a zdraví. Dále byl posouzen vliv na konverzi krmiva, vybrané jatečné ukazatele, kvalitu masa a senzorické vlastnosti.

Čtyři skupiny prasat (n = 10, 5 vepříků a 5 prasniček) v živé hmotnosti 24 kg bylo *semiadlibitně* krmeno 90 dní následujícími dietami: kontrolní skupina (C) - dieta obsahující 3% rybí moučky, pokusná skupina (1) - dieta obsahující 5% tepelně neošetřeného amarantového zrna a 5% sušené nadzemní biomasy amarantu, pokusná skupina (2) - dieta obsahující 5% popovaného (tepelně ošetřeného amarantového zrna) a 5% sušené nadzemní biomasy amarantu, pokusná skupina (3) - dieta obsahující 10% lupinového šrotu. Substitucí animálního proteinu v dietách amarantem (1,2) nebo lupinou (3) nebyl prokázán signifikantní rozdíl ($p > 0.05$) v průměrném denním přírůstku proti kontrolní skupině (C - 0.83 a 0.82, 0.80 a 0.79 kg pro skupiny 1 až 3), neprůkazné rozdíly byli v konverzi krmiva (2.69 až 2.79 kg/kg přírůstku). Testované diety neměly negativní vliv na metabolismus zvířat, signifikantně nižší koncentrace ($p < 0.05$, $p < 0.01$) celkové bílkoviny, glukózy a triacylglycerolu u kontrolních zvířat byly v rozsahu fyziologických limitů. Nebyly zjištěny průkazné rozdíly mezi kontrolními a pokusnými zvířaty v ukazatelích jatečné hodnoty, kvalitě a senzorických vlastnostech masa. Pozitivně byl hodnocen vliv lupiny na křehkost a chuť masa.

Acknowledgements

This work was supported by the Ministry of Agriculture of the Czech Republic, grant MZe No. 0002716201. The authors wish to thank Ing. J. Zajíc from Univit s.r.o., Olomouc, Czech Republic, for valuable recommendations and help concerning compositions of the diets.

References

ACAR N, VOHRA P, BECKER R, HANNERS GD, SAUNDERS RM 1988: Nutritional evaluation of grain amaranth for growing chickens. Poultry Sci **67**: 1166-1173

ANISZEWSKI T, CIESIOLKA D, GULEWICZ K 2001: Equilibrium between basic nitrogen compounds in lupine seeds with differentiated alkaloid content. Phytochemistry **57**: 43-50

ANONYMOUS 2000: Laboratory procedures for testing the feeds additives and premixes (in Czech). ÚKZUZ, Brno, 266 p. (ISBN 80-86051-81-1).

ANONYMOUS 2004: Notice No. 184/2004, Annex 20, Ministry of Agriculture of the Czech Republic (in Czech). 6856-6863.

BATTERHAM ES 1992: Availability and utilization of amino acids for growing pigs. Nutr Res Rev **5**: 1-18

BRESSANI R, CONZALES JM, ZUNIGA J, BREUNER M, ELIAS LG 1987: Yield of amaranth grain representing four species. J Sci Food Afric **38**: 347-356

CANNON JE, BECHTEL PY, EASTER RA, COOK H, McKEITH FH, LESZCZYNSKI DE 1992: Effect of diet containing extruded full- fat soybeans or butter on the growth, compositions and sensory characteristics of pork. J Anim Sci **70**: 3651-3656

CORREA AD, JOKL L, CARLSSON R 1986: Chemical constituents, in vitro protein digestibility, and presence of antinutritional substances in amaranth grains. Arch Latinoam Nutr **36**(2): 319- 326

ČSN 46 61 60: Classification of pig carcasses (in Czech). Prague, ÚNM 2000. 9 p.

DIJKSTRA DS, LINNEMANN AR, VAN BOEKEL TA 2003: Towards sustainable production of protein-rich foods: appraisal of eight crops for Western Europe. PART II: Analysis of the technological aspects of the production chain. Crit Rev Food Sci Nutr **43**(5): 481-506

FERNANDEZ X, MONIN G, TALMANT A 1999: Influence of intramuscular fat content on the quality of pig meat -2. Consumer acceptability on *m. longissimus lumborum*. Meat Sci **53** : 67-72

GDALA J, JANSMAN AJM, van LEEUWEN P, HUISMAN J, VERSTEGEN MWA 1996: Lupins *(L. luteus, L. albus, L. angustifolius)* as a protein source for young pigs. Anim Feed Sci Tech **62**(2-4): 239- 249

GRAJETA H 1999: Effect of amaranth and oat bran on blood serum and liver lipids in rats depending on the kind of dietary fats. Nahrung **43**: 114-117

GREEN AG, ORAM RN 1983: . Anim Feed Sci Tech **9**: 271-282

HOVE EL 1974: Composition and protein quality of sweet lupine seed. J Sci Food Agric **25**: 851-859

IMERI A, FLORES R, ELIAS LG, BRESSANI R 1987: The effect of processing and amino acids supplementation on the protein quality of amaranth (*Amaranthus caudatus*). Arch Latinoam Nutr **37**(1): 161-173

KARAKOZ A 1968: Research in animal husbandry (in Slovak). SVPL Bratislava, 352 p.

LEIKUS R, TRIUKAS K, ·VIRMICKAS G, JU·KIENE V 2004: The influence of various leguminous seeds diets on carcass and meat quality of fattening pigs. Czech J Anim Sci **49**(9): 398- 406

LINNEMANN AR, DIJKSTRA DS 2002: Towards sustainable production of protein-rich foods: appraisal of eight crops for Western Europe. Part I. Analysis of the primary links of the production chain. Crit Rev Food Sci Nutr **42**(4): 377-401

MATOUŠKOVÁ O, CHALUPA J, CÍGLER M, HRUŠKA K 1992: STAT Plus - user manual (in Czech). VÚVeL, Brno, 168 p.

NADĚJE B, KOUCKÝ M, ŠEVČÍKOVÁ S, ADAMEC T, LAŠTOVKOVÁ J 2000: Assessment of boar and barrow meat (in Czech). Czech J Anim Sci **45**: 539-544

OLIVER MA, GISPERT M, DIESTRE A 1993: The effect of breed and halothane sensitivity on pig meat quality. Meat Sci **35**: 105- 118

POND WG, LEHMANN JW 1989: Nutritive value of a vegetable amaranth cultivar for growing lambs. J Anim Sci **67**: 3036-3039

SIMON O, JEROCH H 1999: Feed raw materials part 2 - the up-and- coming legumes. Feed Tech **3**: 29-32

SOKÓL JL, BOBEL BK, FABIJAŃSKA M, BEKTA M 2001: Preliminary results on the influence of amaranth seeds on carcass and meat quality of fatteners. J Anim Feed Sci **10**(Suppl. 2): 203-208

SOMMER A 2003: Soy in animal diets (in Czech). Krmiváfiství 4: 1-4

SOUSA IMN, MORGAN PJ, MITCHELL JR, HARDING SE, HILL SE 1996: Hydrodynamic characterization of lupine proteins: Solubility, intrinsic viscosity, and molar mass. J Agric Food Chem **44**: 3018-3021

SUCHÝ P, STRAKOVÁ E, ZRALÝ Z, PÍSAŘÍKOVÁ B, TRČKOVÁ M, HERZIG I 2005: Lupine seeds as a source of protein (in Czech). Farmář **2**: 37-40

ŠEVČÍKOVÁ S, KOUCKÝ M, LAŠTOVKOVÁ J 2002: Meat performance and meat quality in different genotypes of F_1 generation gilts. Czech J Anim Sci **47**: 395-400

ŠIMEČEK K, ZEMAN L, HEGER J 1993: Nutrient requirement and tables of nutritional values of pig feeds (in Czech). ČAZV Pohořelice, 78 p.(ISBN 80-901598-4-2).

TILLMAN PB, WALDROUP PW 1987: Effects of feeding extruded grain amaranth to laying hens. Poultry Sci **66**: 1698-1701

TLUČHOŘ V 2001: Evaluation of biochemical parameters in veterinary medicine from the standpoint of an individual and a herd (in Czech). Krmivářství **5**: 18-20

TOVAR LR, BRITO E, TAKAHASHI T, MIYAZAWA T, SORIANO J, FUJIMOTO K 1989: Dry heat popping of amaranth seed might damage some of its essential amino acids. Plant Foods Hum Nutr **39**: 299-309

YANEZ E, IVANOVIC D, OWEN DF, BALLESTER D 1983: Chemical and nutritional evaluation of sweet lupines. Ann Nutr Metab **27**: 513-520

ZEMAN L, ŠIMEČEK K, KRÁSA A, et al. 1995: Tables of nutritional values of feeds (in Czech). VÚVZ Pohořelice, 465 p. (ISBN 80-901598-3-4).

ZRALÝ Z, PÍSAŘÍKOVÁ B, HUDCOVÁ H, TRČKOVÁ M, HERZIG I 2004: Effect of feeding amaranth on growth efficiency and health of market pigs. Acta Vet Brno **73**: 437-444

ACTA VET. BRNO 2006, 75: 373-377; doi:10.2754/avb200675030373

Reproductive Performance of Late Pregnant Gilts Treated with Baypamun® before Farrowing

D. POTOČNJAK[1], Ž. PAVIČIĆ[2], H. VALPOTIĆ[3], M. POPOVIĆ[4], LJ. BEDRICA[1], N. VIJTIUK[4], K. VLAHOVIĆ[4], V. MRLJAK[1], R. BARIĆ- RAFAJ[1]

[1]Clinic for Internal Diseases, [2]Department of Animal Hygiene, Environment and Ethology, [3]Department of Animal Nutrition, [4]Department of Biology, Faculty of Veterinary Medicine, University of Zagreb, Zagreb, Croatia

Received November 22, 2005
Accepted March 16, 2006

Abstract

Potočnjak D., Ž. Pavičic, H. Valpotic, M. Popovic, Lj. Bedrica, N. Vijtiuk, K. Vlahovic, V. Mrljak, R. Baric- Rafaj: Reproductive Performance of Late Pregnant Gilts Treated with Baypamun® before Farrowing. Acta Vet Brno 2006, 75: 373-377.

The aim of this study was to investigate whether the production results of pregnant gilts, grown under commercial farm conditions and moved from the sow keeping unit to the prefarrowing unit, could be increased by non-specific immunization with Baypamun® (Bayer, Leverkusen, Germany; BPM), an immune response modifier (IRM). We used three groups of pregnant gilts that obtained different treatments. Non-treated group A served as control; two experimental groups were treated on Day 6, 4 and 2 (group B), or on Day 5, 3 and 1 (group C), respectively, before their transfer from the sow keeping unit to the prefarrowing unit. The experimental gilts received i.m. 2 ml of IRM BPM, i.e. inactivated *Parapoxovis* virus ($1 \times 10^{6.75}$ $TCID_{50}$). Throughout the trial, the numbers of liveborn and stillborn piglets and the duration of farrowing were recorded. Variance analysis with the type of treatment as independent variable showed a significant difference between control (group A) and experimental group B in the number of liveborn piglets ($P < 0.0001$) as well as between group A and group B ($P < 0.0001$) or group C ($P < 0.0001$) in the number of stillborn piglets, respectively. No differences in duration of farrowing between groups were recorded.

Reproduction, stress, immunomodulation, Baypamun®, swine

Mass swine production includes several production stages of which the penultimate stage consists of keeping and housing pregnant sows and gilts in pre-farrowing boxes**.** Each of the production stages requires housing of animals in separate, adequate premises. Thus, upon termination of each production stage the animals are moved from one to another unit. Both the possibility and development of stress in animals are thus increased. Frequent housing changes are critical particularly for pregnant animals that must adjust to the man-made environment. Due to numerous adverse effects the animals can hardly cope with these challenges (Pavičić et al. 2004).

Stress situations may cause changes in homeostatic balance in pregnant animals resulting in compromised production results (Santoro 1996). Exposure of pigs to changed microclimate or social stresses may alter their reproductive functions. For example, each rise in body temperature of 1 - 2 °C causes fertilization disorders and early embryonic death. An increase of intrauterine temperature of 3 °C results in autolysis of embryos. Stress affecting the animals during the first several weeks after insemination strongly affects the reproductive performance of primiparous gilts (increased incidence of abortions or reduced numbers of piglets per litter). The risk is not significant in mid-gestation period, whilst between days 102 and 110 of gestation, heat stress may be the cause of 45% deaths in piglets during parturition (Ivoš et al. 1981). Changes in housing may induce stress reaction in pigs (Barnett et al. 1984; Cronin et al. 1991). Stress hormones may affect the activity of

Address for correspondence:
Doc. dr. sc. Maja Popovic
Department of Biology, Faculty of Veterinary Medicine, University of Zagreb
Heinzelova 55, 10 000 Zagreb, Croatia

Phone: +385-1-2390155
Fax: 385-1-2441390
E-mail: mpopovic@vef.hr
http://www.vfu.cz/acta-vet/actavet.htm

reproductive hormones (Hansen and Curtis 1981) and may also prolong the duration of parturition and increase the number of stillborn piglets.

Numerous agents that modify immune response increase the physiological body resistance by reducing the effects of corticosteroids during stress (Blecha and Charley 1990). One of such substances, Baypamun® (Bayer, Leverkusen, Germany; BPM) has a good efficacy in stimulation of the immune response and control of stress in pigs (Mayr and Brunner 1980; Steinmasl and Wolf 1990; Pavičić et al. 2003).

The aim of this study was to develop an adequate method to reduce stress caused by technology requirements on reproductive performance of gilts, which in turn would improve the reproduction results in intensive swine breeding.

Materials and Methods

The study was performed using pregnant gilts of Swedish Landrace, aged 8 to 9 months. The animals were included in the general production cycle and technological process on the farm together with other animals. The criteria for selection of gilts for insemination comprised their age, minimal body weight of 85 kg, minimal backfat thickness of 20 mm and breed-related phenotype characteristics such as number of teats and their quality. Between the age of 110 and 190 days (according to the breeding technology applied on the farm), all gilts were intramuscularly vaccinated against swine erysipelas, leptospirosis, atrophic rhinitis, Aujezsky disease, parvovirosis, piglet influenza and piglet respiratory and reproductive syndrome (PRRS). The animals were also vaccinated against neonatal colibacillosis and clostridial enterotoxaemia on Day 90 of gestation.

Three groups of 50 selected pregnant gilts each were moved from the sow keeping unit to prefarrowing units where they were housed in groups of 10 into pens. Approximately 5 days (Day 108 of pregnancy) before expected farrowing (between days 112 and 116 of pregnancy) they were moved from prefarrowing unit to farrowing pens. Non-treated group A of pregnant gilts served as control. Treated gilts were intramuscularly injected with 2 ml of BPM solution (containing $1 \times 10^{6,75}$ $TCID_{50}$ of inactivated strain D 1701 of *Parapoxovis* virus) before their transfer to prefarrowing pens.

Based on the treatment they were assigned to two experimental groups which received BPM at either Days 6, 4 and 2 (group B treated at days of pregnancy: 102, 104, 106) or 5, 3, and 1 (group C treated ont Days of pregnancy: 103, 105, 107), respectively. The preparation was administered before feeding the animals (08.30 - 09.00 h).

Two stages of transfer of late pregnant gilts including regrouping and separation may be considered as stressful events accompanying intensive swine production.

The number of live-born and stillborn piglets and duration of parturition (interval between the delivery of the first piglet and completed expulsion of the placenta) were recorded.

The statistical analysis was performed by ANOVA using the STATISTICA 7.1 program (StatSoft 2005).

Results

Table 1. Reproductive indicators in the first-litter gilts treated with BPM at different intervals before their transfer to the pre-farrowing unit on Day 108 of pregnancy (5 days before expected farrowing)

Group of gilts*	Treatment**	Mean ± SD values for		
		No. of live piglets	No. of stillborn piglets	Minutes of duration of parturition
A	None***	9.50 ± 1.18	1.58 ± 0.81	343.60 ± 65.08
B	BPM (at Day 102, 104, 106)	10.60 ± 1,63[a]	0.44 ± 0.58[b]	332.40 ± 49.14
C	BPM (at Day 103, 105, 107)	10.16 ± 1.18	0.66 ± 0.72[b]	348.60 ± 53.22

* Groups comprised 50 gilts each.

** 2 ml of BPM (containing $1 \times 10^{6,75}$ $TCID_{50}$ of inactivated D1701 strain of *Parapoxvirus ovis*) was given i.m. on either Days 102, 104, 106 (group B) or 103, 105, 107 (group C) before the gilts were moved to the prefarrowing unit.

*** 2 ml of saline was given as a placebo (group A).

[a] Significantly ($P < 0.001$) higher No. of live-born piglets than in control gilts.

[b] Significantly ($P < 0.001$) lower No. of stillborn piglets than in control gilts.

Table 1 presents the mean arithmetic values and standard deviations of observed values in separate groups of gilts.

Analysis of variance with the type of treatment as independent variable shows the significant difference in dependent variables of live-born and stillborn piglets (Table 2). Scheffe's test revealed the difference between control (group A) and experimental group B in dependent variable of live piglets ($P < 0.0001$), between controls and experimental group B ($P < 0.0001$) or experimental group C ($P < 0.0001$), respectively, in dependent variable of stillborn piglets.

Table 2. Analysis of variance by the final significance test (F test) with regard to the mean of a variable, e.g. the type of treatment

Indicator	F	df1	df2	Significance*
Live-born piglets	8.431	2	147	0.000
Stillborn piglets	36.464	2	147	0.000
Duration of parturition	1.088	2	147	0.339

*Determining a single variable (e.g. the type of treatment) that discriminates between parameters in the F test.

Discriminant analysis implies significant function (Wilk's Lambda 0.474; chi-square = 109.01; df = 6, $P < 0.0001$). Coefficient of canonical correlation is 0.715. The number of stillborn piglets was the most significant result for all three groups (Table 3). Furthermore, the number of live-born piglets contributes most to the significant discrimination between groups. Duration of farrowing has no influence on the structure of discrimination function.

Table 3. Discriminant function (df) analysis for observed variables with regard to the groups of gilts studied

Group of gilts*	Treatment**	df		
		Live piglets	Stillborn piglets	Duration of parturition
A + B + C	Total gilts* studied	-0.858	1.037	0.109
A	Saline	1.383	-0.064	-
B	BPM (at Day 102, 104, 106)	-1.008	-0.179	-
C	BPM (at Day 103, 105, 107)	-0.375	-0.375	-

* Groups comprised 50 gilts each.

** All the groups of gilts were i.m. injected with either 2 ml of saline (group A) or BPM (containing $1 \times 10^{6,75}$ $TCID_{50}$ of inactivated D1701 strain of *Parapoxvirus ovis*) (group B and group C).

Control group (A) of gilts is on the positive edge of discrimination function, characterized by a smaller number of liveborn and a larger number of stillborn piglets (Table 3). The experimental group B is highly projected at the negative edge of discrimination function characterized by the large number of live-born and small number of stillborn piglets. The experimental group C is closer to zero point.

Discussion

Abundant literature describes the influence of stress factors on the reproductive performance in domestic animals which may occur at different stages of the reproductive cycle, and requires thorough consideration before evaluation of involved variables.

Changes in the reproduction of swine induced by the stress act in accordance with the biological maintenance law, since the animals confronting the stress must survive in order to secure their offspring. Pregnancy represents an additional effect and requirement to the pregnant animal under stressful conditions (Santoro 1996).

Studies performed in 1983 on mice under stress showed that the use of immunomodulators caused reduced secretion of corticosteroids. Baypamun®, an inducer of paraimmunity, has been prepared from the inactivated *Parapoxovis* virus isolated from sheep affected with pustular dermatitis (Büttner et al. 1987; Strube et al. 1989). The efficacy of Baypamun® in the reduction of stress effect and stimulation of immune response in pigs was investigated in numerous studies (Steinmasl and Wolf 1990; Valpotić et al. 1992; Kyriakis et al. 1996; Pavičić et al. 2003).

The use of Baypamun® in pregnant gilts may reduce the losses caused by gastrointestinal syndrome in newborn piglets. Furthermore, studies performed so far provided evidence that administration of Baypamun® to pregnant gilts significantly increases the level of total proteins and immunoglobulins in the blood serum and colostrum of gilts and in the serum of their offspring (Valpotić et al. 1992; Pavičić et al. 2004). Effects of Baypamun® on the reproductive performance of gilts subjected to transport were also studied. It was concluded that paraimmunization of gilts on three occasions during the transport improved their reproductive performance with respect to the number of live-born piglets and the average body weight of piglets at birth (Kyriakis et al. 1996). The stimulation of non-specific immunity of piglets may be helpful in preventing the appearance of post-weaning diarrhoea and wasting pig syndrome (Kyriakis et al. 1998).

The reproductive performance of gilts transported for long distances from three breeding farms to six commercial farms was tested after the use of the immunomodulator Baypamun N. The results indicated that no adverse reactions or adverse effects were noticed related to the treatment with Baypamun N. The treatment improved the reproductive performance of gilts (the proportion of gilts farrowed/total gilts examined, the total number of piglets born, the number of piglets born alive, the body weight of piglets at birth). Furthermore, treatment with Baypamun N reduced the cost of medication per gilt (Saratsis et al. 1999). Since these authors studied the effect of Baypamun N (in form of lyophylisate) on the reproductive performance of gilts following a long distance transport, their results are only in part comparable to ours. The results of our study confirm the beneficial effect of Baypamun® in improving the reproductive performance of gilts by reduction of stress effect. According to measured functions and predictions, we conclude that the optimal timing of Baypamun treatment was used in the first experimental group of gilts.

However, further research is needed in order to cover earlier phases of pregnancy with the treatment of pregnant gilts with either preparation tested (i.e. BPM) or other IRMs. Also, it may be relevant to test and extend our model (Pavičić et al. 2003; 2004) in other technological situations when pregnant sows are regrouped and/or transported from breeding to commercial farms.

Plodnost prasniček ošetřených před porodem v pokročilém stupni březosti Baypamunem®

Cílem studie bylo zjistit, zdali by produkce březích prasniček, odchovaných v podmínkách komerční farmy a přestěhovaných z chovu prasnic na porodnu, mohla být zvýšena nespecifickou imunizací Baypamunem® - (Bayer, Leverkusen, Německo; BPM), imuno-

modulátor (IRM). Použili jsme 3 skupiny březích prasniček, které byly různým způsobem ošetřeny. Neošetřená skupina A sloužila jako kontrola, dvě pokusné skupiny byly ošetřeny následovně: skupina B 6.,4. a 2. den; skupina C 5., 3. a 1. den před přesunem z chovu prasnic na porodnu. Pokusným prasničkám bylo aplikováno 2 ml IRM BPM i.m., inaktivovaného ovčího *Parapoxviru* ($1 \times 10^{6,75}$ $TCID_{50}$). Během pokusu byly zjišťovány počty narozených selat (živých a mrtvých) a byla zaznamenávána délka porodu. Při pokusu byly zjištěny signifikantní rozdíly mezi kontrolní (skupina A) a pokusnou skupinou B v počtech živě narozených selat ($P < 0,0001$) a stejně tak signifikantní rozdíly v počtech mrtvě narozených mezi skupinami A a B ($P < 0,0001$) a skupinou C ($P < 0,0001$). Mezi skupinami nebyly zaznamenány žádné rozdíly v délce porodu.

References

BARNETT JL, CRONIN GM, HEMSWORTH PH, WINFIELD CG 1984: The welfare of confined sows: Physiological, behavioural and production responses to contrasting housing systems and handler attitudes. Ann Rech Vet **15**: 217-226

BLECHA F, CHARLEV B 1990: Rationale for using immunopotentiators in domestic food animals. Adv Vet Sci Comp Med **35**: 3-19

BÜTTNER M, STRUBE W, WOLF G, HOERSTKE M 1987: Parapoxvirus als Induktor unspezifischer Abwehrmechanismen. Tierärztl Umsch **42**: 14-21

CRONIN GM, BARNETT JL, HODGE FM, SMITH JA, McKALLUM TH 1991: The welfare of pigs in two farrowing and lactation environments: Cortisol responses of sows. Appl Anim Behav Sci **32**: 117-127

HANSEN KE, CURTIS SE 1981: Prepartal activity of sows in stall or pen. J Anim Sci **51**: 456-460

IVOŠ J, KRSNIK B, KOVAČEVIC S 1981: The influence of ecological factors on reproduction of pigs. Vet Stn **12**: 63-78

KYRIAKIS SC, ALEXOPOULOS C, GIANNAKOPOULOS K, TSINAS AC, SAOULIDIS K, KRITAS S, TSILOYANNIS V 1996: Effect of a paraimmunity inducer on reproductive performance of gilts. Zentbl Vet Med A **43**: 483-487

KYRIAKIS SC, TZIKA ED, LYRAS DN, TSINAS AC, SAOULIDIS K, SARRIS K 1998: Effect of an inactivated Parapoxvirus based immunomodulator (Baypamun) on post weaning diarrhoea syndrome and wasting pig syndrome of piglets. Res Vet Sci **64**: 187-190

MAYR A, BRUNNER R 1980: Untersuchungen über die Wirksamkeit einer Paraimunisierung zur Bekämpfung von Ferkelaufzuchtverlusten und der enzootischen Pneumonie der Schweinen (Ferkelgrippe). Zentbl Vet Med **27**: 589-598

PAVIČIĆ Ž, VUČEMILO M, TOFANT A, VIJTIUK N, POPOVIĆ M, BALENOVIĆ M, BALENOVIĆ T 2003: Effect of immunostimulator Baypamun on plasma cortisol concentration in gilts regrouped during the late stage of pregnancy. Acta Vet Brno **72**: 509-514

PAVIČIĆ Ž, BALENOVIĆ T, TOFANT A, POPOVIC M, MATKOVIC K, BIUK-RUDAN N 2004: Effect of Baypamun to the level of plasma cortisol in piglets after weaning and transport. Tierärztl Umsch **59**: 450-453

SANTORO P 1996: Lo stress negli animali allevati. Lar Anim Rev **2**: 5-9

SARATSIS P, ALEXOPOULOS C, MAVROMATIS J, TSINAS AC, KYRIAKIS SC 1999: The use of an immunomodulator to enhance the reproductive performance of gilts transported for long distances from breeding to commercial units. Reprod Domest Anim **34**: 67-70

STEINMASL M, WOLF G. 1990: Bindung von Interleukin-2 durch mononukleare Leukozyten des Schweines nach in vitro Stimulation mit verschiedenen Viruspräparaten. J Vet Med **37**: 313-321

STRUBE W, THEIN P, KRETZDORN D, GRUNMACH J 1989: Baypamun: New possibilities for the control of infectious diseases in domestic animals. Vet Med Rev **60**: 3-15

VALPOTIĆ I, VIJTIUK N, RADELJEVIC D, BILIĆ V, KRSNIK B, VRBANAC I, LAUŠIN M 1992: Nonspecific immunization of primiparous sows with Baypamun enhances lacteal immunity in their offspring. Vet arh **63**: 161-172

ACTA VET. BRNO 2006, 75: 379-386; doi:10.2754/avb200675030379

Humoral and Cellular Response of Pheasants Vaccinated against Newcastle Disease and Haemorrhagic Enteritis

S. GRACZYK, A. WIELICZKO[1], A. PLISZCZAK-KRÓL, B. JANACZYK

Division of Pathophysiology at the Department of Pathological Anatomy, Pathophysiology, Microbiology and Forensic Veterinary Medicine;
[1]Department of Epizootiology and Administration with Clinic, Faculty of Veterinary Medicine, Agricultural University of Wroclaw, Wroclaw, Poland

Received December 30, 2005
Accepted April 13, 2006

Abstract

Graczyk S., A. Wieliczko, A. Pliszczak-Król, B. Janaczyk: Humoral and Cellular Response of Pheasants Vaccinated against Newcastle Disease and Haemorrhagic Enteritis. Acta Vet Brno 75, 2006: 379-386.

The purpose of the experiment was to define whether and to what extent can prophylactic vaccinations against Newcastle disease (ND) and haemorrhagic enteritis (HE) affect the humoral and cellular response in pheasants. The evaluation of humoral response was performed on a basis of agglutinin titre after administered antigen and the cellular immunity index was the delayed type hypersensitivity (DTH) reaction. The pheasants were prophylactically vaccinated against Newcastle Disease (ND) on the 1st, 28th and 56th day of life. Moreover, on the 49th day of life, part of the birds was given in the drinking water a vaccine containing the HEV (Haemorrhagic Enteritis Virus). Fourteen days after the HEV vaccination, the birds were intravenously given 0.5 ml of the 10% SRBC (sheep red blood cells) suspension. Simultaneously with the SRBC administration the delayed hypersensitivity test was performed by intradermal administration of phytohaemagglutinin (PHA). It was shown that in pheasants vaccinated with NDV and additionally with HEV, the specific agglutinin anti-SRBC titre was significantly ($p < 0.05$) lower than in birds vaccinated against ND only. It also appeared that, the antibodies resistant to 2-mercaptoethanol were 43% of the total pool of specific anti-SRBC antibodies in the NDV vaccinated birds, whereas in birds vaccinated also with HEV they were 75%. No significant differences were found in the DTH test. Only in the HEV vaccinated pheasants the tendency to increase the wing index value was noted. The results confirm the observations concerning immunosuppressive effects of simultaneous vaccinations. They also indicate that overloading the pheasants with many antigens (ND and HEV vaccination) may weaken the humoral response to administered SRBC.

HEV vaccination, NDV vaccination, anti-SRBC antibody titre, delayed type hypersensitivity (DTH), pheasants

The main role of the immune system is to distinguish between what is its own and what is foreign and to eliminate any threat that may impair homeostasis. The threat is recognized much faster when the factor causing it is known. This knowledge has been used in prevention of animal infectious diseases for years, hence, the use of vaccines containing attenuated pathogenic antigens (Ahmad and Sharma 1993; Pierson and Fitzgerald 2003). The rate and quality of the response depend on animal species and individual cases. In birds, this is influenced by numerous factors, mainly genetic, resulting from natural selection or breeding conditions provided (Kunze et al. 1996; Boa-Amponsem et al. 1999; Talebi et al. 2005). Practically, the key role is attributed to environmental conditions, understood in a very broad sense. As has been shown, the transfer of wild animals into any housing system brings about the threat of increased susceptibility to bacterial and viral diseases. According to many researchers, the increased susceptibility of birds to infectious diseases is an effect of various immunosuppressors (Dohms and Metz 1991; Pierson et al. 1996; Boa-Amponsem et al. 1999; Rautenschlein and Sharma 1999).

Address for correspondence:
prof. dr hab. Stanisław Graczyk
Faculty of Veterinary Medicine
ul. Norwida 31
50-375 Wrocław

E-mail: graczyk@ozi.ar.wroc.pl
http://www.vfu.cz/acta-vet/actavet.htm

Among the viruses deteriorating the reactivity of the immune system in birds there are adenoviruses, such as MSDV - Marble Spleen Disease Virus, commonly found in pheasants. It is a type II avian adenovirus closely related to the haemorrhagic enteritis virus HEV- Haemorrhagic Enteritis Virus and AAS - Avian Adenovirus Splenomegaly (Domermuth et al. 1979; Fitzgerald and Reed 1989; Pierson and Fitzgerald 2003). Infections caused by these viruses are very common. In Poland, the presence of specific anti-HEV anitbodies in ring-necked pheasants (*Phasianus colchicus* L.) was found in 66.7% of flocks under investigation (Wieliczko et al. 2003). These infections usually show no symptoms, but consequently lead to immunosuppression. As a result, the reticuloendothelial cells are damaged, especially in the spleen, which is enlarged and marble-like due to hyperplasia of the white pulp. Morphological changes were also observed in *bursa of Fabricius*, liver, caecal tonsils and kidneys (Gross 1967; Carlson et al. 1974; Trampel 1992). According to Nagaraja et al. (1982), immunosuppression caused by the HE virus is temporary. However, it may cause secondary infections, especially with *E. coli* or coccidia (Sponenberg et al. 1985; Pierson et al. 1996). Some authors report that immunosuppression can also be caused by vaccines containing attenuated viruses (Rautenschlein and Sharma 1999; Kulikova et al. 2004). The mechanisms of immunosuppression are still not well known.

Bearing in mind the facts described above, we evaluated the reactivity of the immune system in pheasants vaccinated against Newcastle disease (ND), and additionally, prophylactically immunized with a vaccine containing a living, attenuated Haemorrhagic Enteritis Virus (HEV).

The criterion for a humoral response assessment in pheasants was the anti-SRBC (sheep red blood cells) haemagglutinin titre. Cellular response was assessed using a delayed type hypersensitivity test (DTH), after intradermal injection of a T-cell mitogen.

Materials and Methods

Experimental procedure

The material taken for the study consisted of 40 pheasants, male and female, purchased from a pheasantry when they were one day old. After several days, the birds were divided into four groups (with the same number of pheasants each) and put in separate cages. All the birds were fed *ad libitum* a complete mixture for pheasants and had free access to water. For the entire experimental period, the birds were fed the same diet and were provided with identical zoohygienic conditions. All the pheasants, one day of age, were prophylactically vaccinated against ND using spray with a Pestos vaccine (Merial), and next, on days 28 and 56 of age, they were given Sotasec vaccine (Merial) in drinking water, according to manufacturers' recommendations. Moreover, 49-day-old birds in groups 3 and 4 were given drinking water containing Dindoral SPF (Merial), a commercial vaccine with Domermuth HEV strain. It is usually recommended for active immunization of turkey broilers (against HEV) and pheasants (against MSDV).

On day 63 of the experiment, i.e. 14 days after HEV vaccination, the birds in groups 2 and 4 were intravenously injected with 0.5 ml of 10% SRBC, suspended in PBS. The experimental procedure comprised the following groups:

Group 1	Group 2	Group 3	Group 4
NDV vaccinated pheasants	NDV + SRBC vaccinated pheasants	NDV + HEV vaccinated pheasants	NDV + HEV + SRBC vaccinated pheasants

Simultaneously with SRBC immunization, the HEV vaccinated and non-vaccinated birds were tested, using a delayed type hypersensitivity test (DTH), according to the procedure described by Parmentier et al. (1998) and Graczyk et al. (1998, 2004). The left wing web was injected with 0.1 cm^3 of PHA - (DIFCO LAB - 1000 $\mu g/cm^3$). The same procedure was used for the right wing, which was injected with PBS (control group).

For comparison, the same dose of PHA was injected intradermally, into the skin, between the third and fourth digits of the right foot. The skin thickness was measured, using a micrometer, exactly in the PBS- and PHA-injected areas, before and 24 h after injection. The magnitude of response was determined by calculating the wing and toe indexes (WI, TI) as the difference in the skin thickness, before and 24 h after intradermal injections.

Serological procedure

The presence of anti-NDV antibodies was performed by the haemagglutination inhibition test (HI test) using an antigen produced by the State Veterinary Institute in Pulawy (Instruction No. GIWZ VII-420/lab-2/2003 on diagnostic tests for Newcastle Disease). The tests were done using blood samples collected from the wing vein of 63-, 70- and 84-day-old birds (i.e. on days 14, 21 and 35 after HEV vaccination). The HI was determined, using twofold increasing dilutions of the serum and a constant dose of ND antigen containing 4 units of HA in 25 ml. The highest dilution of serum, in which haemagglutination was inhibited completely, was accepted as the titre. The HI titre was considered positive when the serum dilution 1:16 and higher inhibited 4 HA antigen units. The results of HI reactivity were accepted as the mean of HI titres for a group expressed in $\log_2$.

The efficacy of HE vaccination was evaluated with regard to the presence of specific anti-HEV antibodies in pheasants' serum obtained from blood collected from the wing vein of 63-, 70- and 84-day-old birds (i.e. 14, 21 and 35 days after HEV vaccination).

The presence of specific anti-HEV antibodies was determined, using precipitation reaction in agar gel (AGP), with the use of pheasant serum and HE antigen (Spafas, USA). The AGP test was performed using Noble Difco agar, in parallel for the investigated sera and also using positive HE Reagent Serum (Spafas, USA). The result was read 24 and 48h after incubation in a humid chamber at 25 °C.

Humoral response to SRBC was evaluated, taking into account the titres of specific agglutinins present in the serum, examined 21 days after HEV vaccination, i.e. 7 days after SRBC immunization. The serum of pheasants, used for determining total anti-SRBC agglutinin titre, was also used to determine the level of antibodies resistant to 2-mercaptoethanol (2-ME-resistant) (Graczyk et al. 2000).

The results obtained in the study were analyzed statistically, using Student's *t*-test. The differences were considered significant at $p < 0.05$.

Results

Table 1 shows the efficiency of prophylactic vaccination, based on the level or presence of specific antibodies.

The presence of specific anti-NDV antibodies was found in 75% to 100% ND-vaccinated pheasants, tested 14 days after HEV vaccination. When the tests were repeated 21 and 35 days after vaccination, the anti-NDV antibodies were present in all the birds. However, the antibody titres were consequently increasing with consecutive blood collections. With the tendency maintained and a lapse of time after vaccination, the absolute value of the titre varied in different groups of birds. The highest titre (6.4 $\log_2$) was found in group 1 (NDV-vaccinated), and a comparable level (5.8 $\log_2$) was found in group 2 (NDV-vaccinated and SRBC-immunized). However, NDV- and HEV-, and to a larger extent, NDV- and HEV-vaccinated + SRBC-immunized pheasants exhibited the antibody titre at a markedly lower level as compared with non-vaccinated birds, i.e. 4.8 $\log_2$ and 4.6 $\log_2$, respectively (Table 1, Fig. 1).

The analysis of the anti-HEV antibody presence in serum showed their lack in birds non-vaccinated with HEV and their presence in all vaccinated birds, but not earlier than 21 days after vaccination (Table 1).

On day 7 after SRBC immunization, the mean specific anti-SRBC agglutinin titre in group 2 was 1 : 588 (g - 512). In contrast, in group 4 (HEV-vaccinated earlier) it was 1 : 154 (g - 135), that is, significantly ($p < 0.05$) lower. Besides, in group 2, over 40% of all specific agglutinins were resistant to 2- mercaptoethanol (IgG), while 2-ME resistant antibodies in HEV- and SRBC-immunized birds (group 4) accounted for 75% (Table 2) .

The mean values of the wing index (WI) and the toe index (TI) are shown in Table 3. The data show that the response to the administered mitogen (PHA) did not result in any significant differences in WI between the control and HEV-vaccinated birds. Similar relations were observed with TI, however, the absolute TI value was twice lower than that of WI (Table 3).

Discussion

The purpose of the present experiment was to study whether and to what extent can prophylactic vaccines containing attenuated NDV and HEV affect the response of the immune system in pheasants, including a cellular response to non-proliferating SRBC and

Table 1. The anti-NDV antibodies titre (log_2 of HI titre) and anti-HEV antibodies presence in blood serum of the experimental pheasants.

		anti-NDV antibodies		anti-HEV antibodies
GROUP	**days after vaccination**	**N - samples number (% of positive)**	**GMT* - (log_2) range**	**AGP****
1-ND	14	8 (100.0)	5.3 (3 – 6)	0/10
	21	8 (100.0)	5.9 (5 – 7)	0/10
	35	8 (100.0)	6.4 (5 – 8)	0/10
2 - ND + SRBC	14	8 (100.0)	5.0 (4 – 6)	0/10
	21	8 (100.0)	5.8 (5 – 7)	0/10
	35	8 (100.0)	5.8 (5 – 7)	0/10
3 - ND + HEV	14	7 (87.5)	4.3 (3 – 5)	6/10
	21	8 (100.0)	4.6 (4 – 5)	10/10
	35	8 (100.0)	4.8 (4 – 5)	10/10
4 - ND + HEV + SRBC	14	6 (75.0)	4.1 (3 – 5)	7/10
	21	8 (100.0)	4.4 (3 – 5)	10/10
	35	8 (100.0)	4.6 (4 – 6)	10/10

Explanations:
*- GMT geometric mean of the HI titre (titre demonstrated as log_2);
** in numerator – the number of positive samples, in denominator – the number of investigated samples

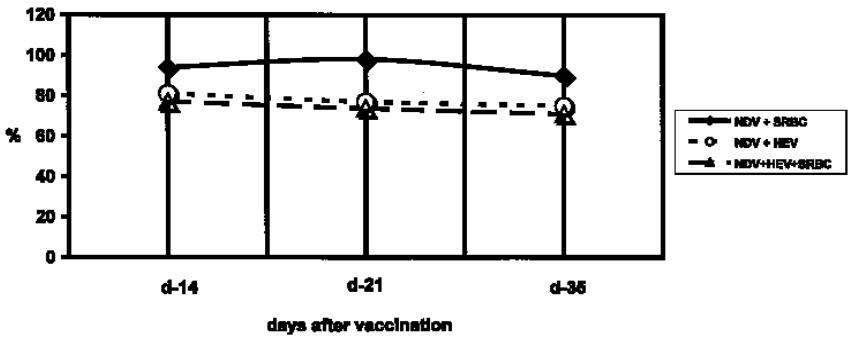

Fig. 1. The anti-NVD antibodies titre in the blood serum of the experimental pheasants (shown as % of the values noted in control group)

Table 2. Anti-SRBC antibodies titre in the blood serum of experimental pheasants.

GROUP	Mean antibody titre (total)	Mean antibody titre (2-ME-resistant)
2 (NDV + SRBC)	588.8 ± 320.42	256 ± 94.53
4 (NDV + HEV + SRBC)	154.67* ± 79.36	116.36* ± 78.73

* - statistically significant difference at $p < 0.05$

Table 3. Mean values of the wing and toe indexes in experimental pheasants (DTH test)

Birds	Wing index (WI) (mm)	Toe index (TI) (mm)
Non-HEV-vaccinated pheasants	2.12 ± 0.57	0.96 ± 0.30
HEV vaccinated pheasants	2.39 ± 0.77	1.18 ± 1.06

* - statistically significant difference at $p < 0.05$

antibody levels. An almost fourfold decrease in anti-SRBC antibody titre observed in pheasants vaccinated with NDV + HEV as compared to NDV-vaccinated birds, accounts for a suppressive action of HEV.

The interaction of various antigens, including living or attenuated vaccines has been studied extensively for many years, but the results obtained show discrepancies (Slowik et al. 1990; Sharma 1994; Pierson et al. 1996; Rautenschlein and Sharma 1999; Ganapathy et al. 2005).

Slowik et al. (1990) found a twofold increase in anti-NDV antibody titre after additional SRBC stimulation of chickens and a decreasing titre after BSA administration. At the same time, they did not observe any impact of NDV vaccination on the specific anti-SRBC agglutinin titre. Ganapathy et al. (2005) reported that SPF chickens vaccinated with living APV and NDV exhibited temporary suppression of APV proliferation and inhibited production of specific antibodies. This corresponded with increased levels of anti-NDV antibody titres in chickens. Some authors even suggest that administration of various vaccines at short-time intervals may result in temporary changes in the immune system, which may be favourable for the development of secondary infections (Pierson et al. 1996; Rautenschlein and Sharma 1999).

The decreased anti-SRBC antibody titre observed in HEV-vaccinated pheasants is therefore in agreement with the observations of the authors cited above, and besides, it shows that simultaneous vaccination can enhance pathogenicity of the vaccines administered. It is also worth noting that anti-NDV antibody titre in NDV + HEV-vaccinated pheasants was over 20% lower than that observed in the pheasants vaccinated only with NDV. Additional SRBC stimulation of the birds enhanced the suppressive effect of HEV, which suggests that immunosuppression is not directed towards a definite antigen. On the other hand, simultaneous vaccination did not have a significant impact on antibody levels, observed in consecutive blood samples after vaccination. Due to the fact that our experiment was focused only on the presence of anti-HEV antibodies, excluding the titre, it was impossible to assess the influence of NDV on anti- HEV antibody titre. Other interesting results obtained in our study were connected with a changing quality of anti-SRBC antibodies, since HEV-vaccinated birds exhibited augmented percentages of 2-ME resistant antibodies. A direct mechanism responsible for the change of class of the synthetized antibodies is, at this stage of research, difficult to explain.

The studies of HE pathogenesis show that the immune system, which is connected with antibody production, plays an important part in the development of a disease. Suresh and Sharma (1995) studied turkeys inoculated with HEV and found a significant depletion of IgM + cells. On the other hand, the studies of immunosuppressive actions of viruses show that depletion of B lymphocytes, occurring after chemical bursectomy, results in a preventive effect of experimental infection of birds (Fitzgerald and Reed 1991). In our study, no lymphatic cells were evaluated, but we can presume that a decreased production of antibodies was due to temporary changes in CD4 CD8 populations, as reported by Suresh and Sharma (1995). It seems quite likely that this was also connected with the effect of vaccines on spleen cells, since the impact of the spleen on immunological response is remarkable (Graczyk et al. 1998, 2003; Rautenschlein and Sharma 1999).

Clinical observations and the studies of cyclosporine-treated birds suggest that T lymphocytes, and at the same time, cellular response can play an important role in the pathogenesis of MSD and responses to MSDV infections (Fitzgerald et al. 1995). The reactivity of T-cells in birds is evaluated using delayed-type hypersensitivity response to the administered T-cell mitogen (Parmentier et al. 1998; Boa-Amponsem et al. 1999).

The analysis of cellular response with the use of DTH test did not show any significant differences between HEV-vaccinated and non-vaccinated pheasants. At the same time, the results obtained in the study are in agreement with the suggestions of other authors who claim that the part of the body into which a mitogen is administered is of no importance. With significant differences in extreme values of the index noted, only some increasing tendencies were observed in WI and TI of HEV-vaccinated pheasants. These results are in agreement with the reports of Fitzgerald et al. (1992), who found that pheasants which had a contact with an MSD virus, showed a changed capability of T-lymphocytes for transformations. In our experiment, cellular response was assessed only once, 14 days after HEV inoculation, therefore, it is difficult to say whether the results of the DTH test reflect constant reactivity of the system, or they just illustrate a transient phase of the virus activity during immunogenesis.

We can state that the results obtained in our experiment are in concurrence with those obtained by other authors who studied the immunosuppressive effects of simultaneous vaccinations. Besides, they also show that consecutive overloading of pheasants with antigens can result in a reduced response of the birds to other antigens, which should be taken into consideration when scheduling prophylactic vaccinations for this species.

Humorální a celulární imunitní odpověď bažantů vakcinovaných proti Newcastleské nemoci a hemorrhagické enteritidě

Cílem pokusu bylo určit zda a v jaké míře může preventivní vakcinace proti Newcastleské chorobě (ND) a hemorrhagické enteritidě (HE) ovlivnit humorální a celulární imunitní odpověď u bažantů. Vyhodnocení míry humorální imunitní odpovědi bylo provedeno na základě titru aglutininu po podání antigenu, celulární imunita byla hodnocena alergenodiagnostickým testem (DTH). Bažanti byli 1., 28. a 56. den po vylíhnutí očkováni proti Newcastleské nemoci (ND). Kromě této vakcinace byla části ptáků 49. den po vylíhnutí podána vakcína proti HEV v pitné vodě (virus hemorrhagické enteritidy). Čtrnáct dní po vakcinaci proti HEV bylo ptákům intravenózně podáno 0,5 ml 10% SRBC (suspenze ovčích červených krvinek). S podáním SRBC byla současně provedena alergenodiagnostika intradermální aplikací fytoheaglutininu (PHA). U bažantů vakcinovaných proti NDV i HEV byl titr specifických anti-SRBC aglutininů signifikantně nižší ($p < 0,05$) než u těch vakcinovaných pouze proti ND. Též se ukázalo, že 43% ze všech specifických anti-SRBC protilátek u ptáků vakcinovaných proti NDV bylo rezistentních k 2-merkaptoethanolu, zatímco u ptáků vakcinovaných proti HEV jich bylo rezistentních 75%. U alergenodiagnostického testu nebyly

zaznamenány žádné signifikantní rozdíly. Jen u bažantů vakcinovaných proti HEV byla zjištěna tendence ke zvýšené hodnotě odpovědi. Výsledky potvrzují pozorování o imunosupresivním efektu souběžné vakcinace. Také ukazují, že přesycení/přetížení bažantů mnoha antigeny (ND a HEV vakcinace) může oslabit humorální imunitní odpověď na podané SRBC.

References

AHMAD J, SHARMA JM 1993: Protection against haemorrhagic enteritis and Newcastle disease in turkeys by embryo vaccination with monovalent and bivalent vaccines. Avian Dis **37**: 485-491

BOA-AMPONSEM K, LARSEN CT, DUNNINGTON EA, SIEGEL PB 1999: Immunocompetence and resistance to marble spleen disease of broiler- and layer-type pure lines of chickens. Avian Pathol **28**: 397-384

CARLSON HC, AL-SHEIKLY F, PETTIT JR, SEAWRIGHT GL 1974: Virus particles in spleens and intestines of turkeys with haemorrhagic enteritis. Avian Dis **18**: 67-73

DOHMS JE, METZ A 1991: Stress mechanisms of immunosuppression. Vet Immunol Immunopat **30**: 89-109

DOMERMUTH CH, GROSS WB, SCHWARTZ LD, MALLISON ET, BRITT L 1979: Vaccination of ring-necked pheasant for marble spleen disease. Avian Dis **23**: 30-38

FITZGERALD SD, FITZGERALD AL, REED WM, BURNSTEIN T 1992: Immune function in pheasants experimentally infected with marble spleen disease virus. Avian Dis **36**: 410-414

FITZGERALD SD, REED WM, FURUKAWA AM, ZIMELS E, FUNG L 1995: Effect of T-lymphocyte depletion on the pathogenesis of marble spleen disease virus infection in ring-necked pheasants. Avian Dis **39**: 68-73

FITZGERALD SD, REED WM 1989: A review of marble spleen disease of ring-necked pheasants. J Wildlife Dis **25**: 455-461

FITZGERALD SD, REED WM 1991: Pathogenesis of marble spleen disease in bursectomized and non-bursectomized ring-necked pheasants following oral inoculation with cell-culture-propagated virus. Avian Dis **35**: 579-584

GANAPATHY K, CARGILL P, MONTIEL E, JONES RC 2005: Interaction between live avian pneumovirus and Newcastle disease virus vaccines in specific pathogen free chickens. Avian Pathol **34**: 297-302

GRACZYK S, KURYSZKO J, MADEJ J 2003: The reactivity of spleen germinal centres in immunized and ACTH treated chickens. Acta Vet Brno **72**: 523-531

GRACZYK S, MADEJ J, PLISZCZAK-KRÓL A 1998: The influence of splenectomy on humoral and cellular immune response and morphology of central lymphatic organs in the chickens immunized by different type of antigens. Sci Lett (Wroclaw, Agric. University) **58**: 7-15 (in Polish)

GRACZYK S, SLOWIK J, ORDA J 2000: The influence of Tolpa Peat Preparation (TPP) on the course of humoral response in chickens fed with fodder containing animal protein of livex origin. Bulletin of the Polish Academy of Sciences Biological Sciences **48**: 293-304

GRACZYK S, ZAWADZKI W, CZERSKI A, PLISZCZAK-KRÓL A, KOTONSKI B 2004: Delayed type hypersensitivity reaction (DTH) in the turkeys treated with beta adrenergic antagonist and ACTH. EJPAU Electronic Journal of Polish Agricultural Universities, Series Veterinary Medicine **7** (2)

GROSS WB 1967: Lesions of haemorrhagic enteritis. Avian Dis **11**: 684-693

KULIKOVA L, JURAJDA V, JURANOVA R 2004: Effects of infectious bursal disease vaccination strains on the immune system of leghorn chickens. Acta Vet Brno **73**: 205-209

KUNZE KL, FITZGERALD SD, RICHARD A, BALANDER R, REED WM 1996: Variation in response of four lines of ring-necked pheasants to infection with marble spleen disease virus. Avian Dis **40**: 306-311

NAGARAJA KV, EMERY DA, PATEL BJ, POMEROY BS, NEWMAN JA 1982: In vitro evaluation of B-lymphocyte function in turkeys infected with haemorrhagic enteritis wirus. Am J Vet Res **43**: 502-504

PARMENTIER HK, REILINGH GD, NIEUWLAND MGB 1998: Kinetic and immunohistochemical characteristics of mitogen-induced cutaneous hypersensitivity in chickens selected for antibody responsiveness. Vet Immunol Immunopat **66**: 367-376

PIERSON FW, FITZGERALD SD 2003: Haemorrhagic enteritis and related infections. In: Saif Y.M, Diseases of Poultry 11th ed., Iowa Press, pp. 237-247.

PIERSON FW, LARSEN CT, DOMERMUTH CH, 1996: The production of colibacillosis in turkeys following sequential exposure to Newcastle disease virus or Bordetella avium, avirulent haemorrhagic enteritis virus, and Escherichia coli. Avian Dis **40**: 837- 840

RAUTENSCHLEIN S, SHARMA JM 1999: Response of turkeys to simultaneous vaccination with haemorrhagic enteritis and Newcastle disease viruses. Avian Dis **43**: 286-292

SHARMA JM 1994: Response of specific-pathogen-free turkeys to vaccinates derived from marble spleen disease virus and haemorrhagic enteritis virus. Avian Dis **38**: 523-530

SLOWIK J, GRACZYK S, GOLNIK W, PAWESKA J 1990: Immunological response of bursectomized and non-bursectomized chickens inoculated with Newcastle disease virus (NDV), after administration of sheep red blood cells (SRBC) or bovine serum albumin (BSA). Pol Arch Wet **30**: 101-112 (in Polish)

SPONENBERG DP, DOMERMUTH CH, LARSEN CT 1985: Field outbreaks of colibacillosis of turkeys associated with haemorrhagic enteritis virus. Avian Dis **29**: 838-842

SURESH M, SHARMA JM 1995: Haemorrhagic enteritis virus induced changes in the lymphocyte subpopulations in turkeys and the effect of experimental immunodeficiency on viral pathogenesis. Vet Immunol Immunopat **45**: 139-150

TALEBI A, POURBAKHSH SA, DOROSTKAR K 2005: Effects of vaccination routes against IB on performance and immune responses of broiler chickens. Int J Poultry Sci **4**: 795-798

TRAMPEL DW, METEYER CU, ARTUR A, BICKPORD AA 1992: Haemorrhagic enteritis virus inclusions in turkey renal tubular epithelium. Avian Dis **36**: 1086-1091

WIELICZKO A, TOMANEK B, KUCZKOWSKI M 2003: Prevalence of infectious diseases in ring-necked pheasant flocks in Poland. Pol J Vet Sci **6**: 177-182

Dose-dependent Effect of T-2 Toxin on the Immunity against Newcastle Disease Virus in Chickens

M. WEBER[1], J. FODOR[2], K. BALOGH[1], M. ERDÉLYI[1], M. MÉZES[1]

[1]Department of Nutrition, Szent István University, Gödöllö, Hungary
[2]Department of Animal Physiology, Faculty of Animal Science, Kaposvár University, Kaposvár, Hungary

Received January 13, 2006
Accepted June 30, 2006

Abstract

Weber M., J. Fodor, K. Balogh, M. Erdélyi, M. Mézes: Dose- dependent Effect of T-2 toxin on the Immunity against Newcastle Disease Virus in Chickens. Acta Vet. Brno 2006, 75: 387-391.

The effect of 2.35 (a) and 4.18 (b) mg·kg^{-1} feed T-2 toxin dose for 14 days on the haemagglutination inhibition titres against Newcastle disease virus was investigated in broiler chickens. The animals were divided into four groups and two separate experiments were carried out (a, b): (1) intact control group; (2) birds were fed with T-2 toxin contaminated feed and not vaccinated; (3) repeatedly vaccinated (on day 23 of age) control group which received uncontaminated feed; (4) birds were both repeatedly vaccinated and fed the T-2 toxin contaminated diet. Blood samples, from which sera titres were measured, were taken on days 7 and 14 of the experiments. It was found that heamagglutination titres were different in the two experiments even in the control (1) group because of the different efficiency of the first immunization at the hatchery. Titres on day 7 showed increases in all groups except for the group fed lower T-2 contaminated diet (a, group 2) but during the second week they increased only in the groups fed the diet with a lower dose of T-2 toxin. On the contrary, a higher dose of T-2 toxin contamination of the diet resulted in a dramatic decrease during the second week (b, groups 2 and 4). The results suggested that contrary to most of the previously published data, feeding of T-2 toxin contaminated feed with an amount of 2.35 mg.kg^{-1} did not decrease, but increase the antibody formation against attenuated Newcastle disease virus even without a second vaccination on day 1 of the experiment, whereas a higher amount of T-2 toxin (4.18 mg·kg^{-1}) decreased to day 14 after the repeated vaccination.

T-2 toxin, Newcastle disease, heamagglutination inhibition, immunity, chicken

Mycotoxins are a group of structurally diverse fungal secondary metabolites that elicit a wide spectrum of toxicological effects, such as different rate of immunosuppression (Surai and Dvorska 2005). This effect is caused by the mycotoxins, e.g. T-2 toxin, and its metabolites mainly in the thymus and spleen (Lafarge-Frayssinet et al. 1990). The T-2 toxin impairs the natural defence mechanism, including altered parameters of humoral-mediated immunity, depressed T or B lymphocyte activity (Oswald and Comera 1998) and precursors of B cells (Holladay et al. 1995). Splenic (Kamalavenkatesh et al. 2005), and thymic (Nagata et al. 2001) CD+8 and CD+4 lymphocytes represent highly sensitive targets of T-2 toxin exposure (Holladay et al. 1995). The immunosuppression of the organism results in dose-dependent, significant decrease in antibody formation in pigs (Rafai et al. 1995), and also a manifestation of different diseases, such as swine dysentery (Sándor and Ványi 1990). Feeding T-2 toxin contaminated diet (5 mg·kg^{-1}) for 21 days caused reduced neutralizing antibody production in response to a single subcutaneous dose of vaccine against haemorrhagic gastroenteritis (Rafai et al. 1989). On the contrary, a long-term treatment, a single high oral dose of T-2 toxin (3 mg·kg^{-1} b.w.) but not 14-days pre-treatment with a low dose (0.75 mg·kg^{-1} b.w.) have modulatory effects on the respiratory burst activity of macrophages and cell-mediated immune system and cause enhancement of resistance to *E. coli* and *Staphylococcus aureus* in mice (Cooray

Address for correspondence:
Miklós Mézes
Department of Nutrition, Szent István University
H-2103 Gödöllö, Páter Károly u. 1., Hungary

Phone: +36-28 410 735
Ffax: +36-28 410 804
E-mail: Mezes.Miklos@mkk.szie.hu
http://www.vfu.cz/acta-vet/actavet.htm

and Jonsson 1990). On the contrary, T-2 toxin treatment at 6 and 1 days before BCG vaccination against murine pulmonary tuberculosis did not alter the efficacy of the vaccine (Ziprin and McMurray 1989). In the case of newly-hatched chickens, a 6-day pre-treatment with T-2 contaminated feed had a profound negative effect on the ability of the birds to resist salmonellosis, as measured by survival (Ziprin and Elissalde 1990). It was concluded (Ram et al. 1990) that T-2 toxin either at low or at high concentrations of single treatment stimulated lymphocytes. However, during a long-term treatment particularly with high doses of toxin, lymphocyte response was inhibited. No significant effect of T-2 toxin up to the concentration of 1 $mg \cdot kg^{-1}$ feed was observed on antibody production to antigens administered by enteral or parenteral routes in chicken (Sklan et al. 2001) and turkey (Sklan et al. 2003). No significant effect was found in turkey poults and chickens fed T-2 toxin contaminated feed even at a 10 $mg \cdot kg^{-1}$ concentration (Richard et al. 1978). In respect of haemagglutination inhibition antibody titre to Newcastle disease, the data on experimental results showed that a daily oral dose of T-2 toxin at a dose of 1.25 $mg \cdot kg^{-1}$ body weight (approximately 12.5 $mg \cdot kg^{-1}$ feed) for one week did not affect the titre at the 4th week of age (Hossain et al. 1987); on the contrary, 3 $mg \cdot kg^{-1}$ T-2 toxin contaminated feed reduced the titre in a five-week experiment (Raju and Devegowda 2002).

Materials and Methods

Eighty chickens of the same genotype (ROSS 308 broiler cockerels), weighing 1.12 ± 0.23 kg, were used in the experiment. The animals were placed in cages during the trials. The temperature (23 ± 2 ºC) of the trial room was controlled in accordance with the needs of the broiler. Feed and drinking water was available *ad libitum.*

The experimental animals (initial age: 23 days) were divided into four groups of 20 birds each in experiment "a" and experiment "b". With the exception of the intact control (1) and the vaccinated control (3) groups, chicken feed was contaminated with T-2 toxin at a concentration of 2.35 (a) and 4.18 (b) $mg \cdot kg^{-1}$ feed and was administered for 14 days. Although there is no official regulation in the European Union for the maximum allowed T-2 toxin content in the feeds, a guideline limit of 0.5 $mg \cdot kg^{-1}$ T-2 toxin has been proposed (Eriksen and Pettersson 2004). T-2 toxin contamination in the present experiment was much higher than the maximum guideline limit level.

The partially purified toxin preparation was dissolved in acetone and then sprayed onto the compound feed (100 ml/50 kg of feed). T-2 toxin was produced experimentally on maize by *Fusarium sporotrichoides,* strain NRRL 3299 (Agricultural Research Service Culture Collection, National Center for Agricultural Utilization Research, Peoria, IL) as described below.

Maize was prepared in 4.2-liter wide-mouthed glass fruit jars with a cover (cotton plug between 2 linen rags) by autoclaving 800 g yellow maize kernels in 400 ml water for 2 h at 121 °C, after soaking overnight. The prepared maize was inoculated with the 8-day-cultivated *F. sporotrichoides* strain NRRL 3299. The culture was incubated in darkness at 24 °C for a week, than at 8 °C for 2 weeks. After opening, the fungus-infected maize was dried at room temperature for several days, and then it was ground. The homogenized fungal culture contained T-2 toxin at a concentration of 1180.5 $mg \cdot kg^{-1}$. Extraction and purification of toxin were carried out according to Burmeister (1971). Purity of the T-2 toxin preparation was determined by the HPLC method and exceeded 95% (Békési et al. 1997). The T-2 toxin content of the feed was measured by the HPLC technique (AOAC 1990). The amount of related trichothecenes, HT-2, T-2 triol and T-2 tetraol, were under the limit of detection (< 0.04 $mg \cdot kg^{-1}$).

The animals in the vaccinated groups received attenuated Newcastle disease vaccine in the form of eye drops (NobilisR ND Clone 30, Intervet International bv, Boxmeer) on day 1 of the experiment according to the producer's instruction.

The four groups (Table 1) in both experiments (a and b) were as follows: (1) intact control group; (2) birds fed T-2 toxin contaminated feed and not vaccinated; (3) vaccinated control group which received uncontaminated feed; (4) birds were both vaccinated and fed a T-2 toxin contaminated diet.

Blood samples were taken on days 7 and 14 from the cubital vein, and blood serum was analysed using haemagglutination inhibition (HI) test according to Balla (1980).

The average haemagglutation titres were calculated with geometrical mean using MS Excel 7.0 software and statistically evaluated based on the $\log_2$ transformation of the dilution rate as proposed by Balla (1980) by ANOVA analysis using Statistica® 4.5 (Statsoft Inc., 1993) software.

The experiment was approved by the Animal Experimental Committee of the Faculty of Agricultural and Environmental Sciences of Szent István University (3/2003 MKK).

Table 1. Treatments given in the different groups

Group	Treatment		
	T-2 toxin concentration (mg·kg^{-1} feed)		Vaccination
	a	b	
1	-	-	-
2	2.35	4.18	-
3	-	-	+
4	2.35	4.18	+

Results

The haemagglutination inhibition titres (Table 2) were different in the two experiments even in the control group (1), i.e. the first immunization at the hatchery had different efficacy. Haemagglutination titres were significantly different among the groups (Table 3) as an effect of vaccination, and also in groups fed a T-2 toxin contaminated diet at a lower dose (2.35 mg·kg^{-1}). It was the highest in the group which was fed a T-2 toxin contaminated diet and repeatedly vaccinated (a, group 4) on day 7. On day 14 both groups fed a lower T-2 toxin contaminated diet showed a dramatic and significant increase in the haemagglutination titres both without (a, group 2) and with repeated vaccination (a, group 4). In contrast, the haemagglutination titres did not differ significantly (Table 3) by feeding a T-2 toxin contaminated diet at a higher dose (4.35 mg·kg^{-1}) on day 7 but it was also the highest in the group fed T-2 toxin contaminated diet and repeatedly vaccinated (Table 2) against Newcastle disease virus (b, group 4). On day 14 after the repeated vaccination haemagglutination titres were significantly different among the groups but showed opposite tendency in the groups fed T-2 toxin contaminated diet at a higher dose than at a lower one (Table 2). Namely the haemagglutination titre decreased in these groups as compared to the value on day 7 either without (b, group 2) or with repeated vaccination (b, group 4).

Table 2. Effect of T-2 toxin contaminated feed and/or vaccination against Newcastle disease on haem agglutination inhibition titres in blood sera of chicken

Group	Period after repeated vaccination (days)	HAG titre	
		Geometric mean	$\log_2$ mean ± SD
Experiment (a)- 2.35 mg·kg^{-1} T-2 toxin in feed			
1	7	3.2	1.68 ± 0.00
	14	44.6	5.48 ± 4.38
2	7	2.7	1.43 ± 0.96
	14	289.6	8.18 ± 1.00
3	7	18.1	4.18 ± 3.00
	14	172.2	7.43 ± 1.50
4	7	102.4	6.68 ± 1.22
	14	409.6	8.68 ± 0.00
Experiment (a) - 4.18 mg·kg^{-1} T-2 toxin in feed			
1	7	2.0	1.00 ± 0.00
	14	16.0	4.00 ± 4.12
2	7	9.2	3.20 ± 0.44
	14	2.0	1.00 ± 3.04
3	7	57.0	8.38 ± 0.57
	14	445.7	8.80 ± 0.83
4	7	128.0	7.00 ± 1.26
	14	45.3	5.50 ± 1.51

Table 3. ANOVA analysis of HAG titres expressed in $\log_2$ of the dilution rate

Period after the repeated vaccination (days)	Toxin dose (mg/kg feed)	Mean square	Mean square error	F(df1.2) 1.9	p-level
7	2.35	75	7.91453	9.476242	0.013175
14	2.35	432	1.230769	351	0.0000000161
7	4.18	3	11.65812	0.257331	0.624156
14	4.18	108	12.76923	8.457831	0.017361

Discussion

Mycotoxins, particularly trichothecene toxins, were declared as immunosuppressive compounds in animals (Surai and Dvorska 2005), but certain previous experiments did not find a significant effect of T-2 toxin on antibody production in reaction to antigens administered by enteral or parenteral routes in chickens (Sklan et al. 2001) and turkeys (Sklan et al. 2003) up to 1 mg·kg^{-1} feed. The same non-significant effect was found in turkey poults and chickens fed T-2 toxin contaminated feed even at a 10 mg·kg^{-1} concentration (Richard et al. 1978). In respect of haemagglutination inhibition antibody titre to Newcastle disease, data on the experimental results showed that T-2 toxin with 1.25 mg·kg^{-1} body weight (approximately 12.5 mg·kg^{-1} feed) did not affect the titre at the 4th week of age (Hossain et al. 1987); on the contrary, 3 mg·kg^{-1} T-2 toxin contaminated feed reduced the titre in a five-week experiment (Raju and Devegowda 2002).

The results of the present study showed that a T-2 toxin contaminated feed has a marked effect on the immunity against Newcastle disease in a dose- and time-dependent manner. The immune system was activated and haemagglutination titres were increased, by a lower level of T-2 toxin (2.35 mg·kg^{-1}) loading for 14 days even without repeated vaccination on day 23 of age. On the contrary, using a higher dose (4.18 mg·kg^{-1}) of contamination of T-2 toxin haemagglutatination titres increased up to day 7, but decreased dramatically during the next week. These results suggest that the effect of T-2 toxin on the immune response in chicken depends on the age of animals, amount of mycotoxin, also the period of feeding the T-2 toxin contaminated diet. Summarizing the results, it can be concluded that the well-known immunosuppressive effect manifested only at a high level of contamination (4.18 mg·kg^{-1}) and after a longer period (14 days) of time.

Účinek různých dávek T-2 toxinu u kuřat na tvorbu protilátek proti viru Newcastleské nemoci (ND)

U brojlerových kuřat byl zkoumán vliv T-2 toxinu, v dávce 2,35 (a) a 4,18 (b) mg·kg^{-1} krmiva po dobu 14ti dní, na titry protilátek v hemaglutinačně inhibičním testu u viru Newcastleské choroby. Ptáci byli rozděleni do dvou skupin a byly provedeny dva samostatné pokusy (a,b): (1) intaktní kontrolní skupina; (2) ptákům bylo podáno krmivo s T-2 toxinem, ale nebyli vakcinováni, (3) kontrolní skupina opakovaně vakcinovaných (23. den po vylíhnutí), která nedostala žádné krmivo s T-2 toxinem; (4) ptáci byli vakcinováni a bylo jim podáno krmivo s T-2 toxinem. Sedmý a 14. den experimentu byly odebrány vzorky krve k zjištění titrů protilátek ze séra. Při každém ze dvou pokusů byly zjištěny různé hemaglutinační titry a to i u kontrolní (1) skupiny vzhledem k rozdílné účinnosti první imunizace v líhni. Sedmý den vzrostly titry u všech skupin, kromě té krmené směsí s nižší dávkou T-2 toxinu (a, skupina 2). Druhý týden se titry zvýšily už jen u skupiny, které byla podána nižší dávka T-2 toxinu. Naproti tomu vyšší dávka T-2 toxinu v krmivu vedla k významnému snížení během druhého týdne (b, skupiny 2 a 4). Výsledky ukazují, že navzdory mnoha předešlým publikacím, krmení kuřat krmivem kontaminovaným T-2 toxinem v množství

2,35 mg·kg^{-1} nesnížilo, ale naopak zvýšilo tvorbu protilátek proti atenuovanému viru Newcastleské nemoci dokonce i bez druhé vakcinace v 1. den pokusu, zatímco vyšší množství T-2 toxinu (4,18 mg·kg^{-1}) ji do 14. dne po opakované vakcinaci snížilo.

References

AOAC 1990: Official methods of analysis of the Association of Official Analytical Chemists, 15th ed., Association of Official Analytical Chemists, Arlington, Va.

BALLA L 1986: Use of standardized haemagglutination inhibition (HI) test for checking the immunity against Newcastle disease. I. Experiments to standardize the HI test (In Hungarian). Magy Állatorv Lapja **41**: 98-103

BÉKÉSI L, HORNOK S, SZIGETI G, DOBOS-KOVÁCS M, SZÉLL Z, VARGA I 1997: Effect of F-2 and T-2 fusariotoxins on experimental *Cryptosporidium baileyi* infection in chickens. Int J Parasitol **27**:1531-1536

BURMEISTER HR 1971: T-2 toxin production by *Fusarium tricinctum* on solid substrate. Appl Microbiol **21**: 739-742

COORAY R, JONSSON P 1990: Modulation of resistance to mastitis pathogens by pre-treatment of mice with T-2 toxin. Food Chem Toxicol **28**: 687-692

ERIKSEN GS, PETTERSSON H 2004: Toxicological evaluation of trichothecenes in animal feed. Anim Feed Sci Technol **114**: 205- 239

HOLLADAY SD, SMITH BJ, LUSTER MI 1995: B-lymphocyte precursor cells represent sensitive targets of T-2 mycotoxin exposure. Toxicol Appl Pharmacol **131**: 309-315

HOSSAIN MI, GUPTA RKP, BHATIA KC, MISHRA UK 1987: Haemagglutination inhibition titres to Newcastle disease virus in T-2 mycotoxin administered chicks. Bangladesh Vet J **21**: 99-102

KAMALAVENKATESH P, VAIRAMUTHU S, KALACHANDRAN C, MANOHAR BM, RAJ GD 2005: Immunopathological effect of the mycotoxins cyclopiazonic acid and T-2 toxin on broiler chicken. Mycopathologia **159**: 273-279

LAFARGE-FRAYSSINET C, CHAKOR K, LAFONT P, FRAYSSINET C 1990: Transplacental transfer of T-2 toxin: pathological effects. J Exp Pathol Toxicol Oncol **10**: 64-66

NAGATA T, SUZUKI H, ISHIGAMI N, SHINOZUKA J, UETSUKA K, NAKAYAMA H, DOI K. 2001: Development of apoptosis and changes in lymphocytes subsets in thymus, mesenteric lymph nodes and Peyer's patches of mice orally inoculated with T-2 toxin. Exp Toxicol Pathol **53**: 309-315

OSWALD JP, COMERA C 1998: Immunotoxicity of mycotoxins. Rev Med Vet **149**: 585-590

RAJU MVLN, DEVEGOWDA G 2002: Esterified-glucomannan in broiler chicken diets contaminated with aflatoxin, ochratoxin and T-2 toxin: evaluation of its binding ability (in vitro) and efficacy as immunomodulator. Asian-Australasian J Anim Sci **15**: 1051-1056

RAFAI P, TUBOLY S, TURY E 1989: Effect of T-2 fusariotoxin on adrenocortical function and some immunological parameters in growing pigs (In Hungarian). Magy Allatorv Lapja **44**: 299-303

RAFAI P, TUBOLY S, BATA A, TILLY P, VÁNYI A, PAPP Z, JAKAB L, TURY E 1995: Effect of various levels of T-2 toxin in the immune system of growing pigs. Vet Rec. **136**: 511-514

RAM GC, RAO MVS, BANSAL MP 1990: Inhibition of blastogenesis in cattle lymphocytes by T-2 toxin and its restoration with interleukin-2 (IL-2). Ind J Anim Sci **60**: 273-278

RICHARD JL, CYSEWSKI SJ, PIER AC, BOOTH GD (1978): Comparison of effects of dietary T-2 toxin on growth, immunogenic organs, antibody formation, and pathologic changes in turkeys and chickens. Am J Vet Res **39**: 1674-1679

SANDOR G, VANYI A 1990: Mycotoxin research in the Hungarian Central Veterinary Institute. Acta Vet Hung **38**: 61-68

SKLAN D, KLIPPER E, FRIEDMAN A, SHELLY M, MAKOVSKY B 2001: The effect of chronic feeding of diacetoxyscirpenol, T-2 toxin, and aflatoxin on performance, health and antibody production in chicks. J Appl Poult Res **10**: 79-85

SKLAN D, SHELLY M, MAKOVSKY B, GEYRA A, KLIPPER E, FRIEDMAN A 2003: The effect of chronic feeding of diacetoxyscirpenol and T-2 toxin on performance, health, small intestinal physiology and antibody production in turkey poults. Br Poult Sci **44**: 46- 52

SURAI PF, DVORSKA JE (2005): Effects of mycotoxins on antioxidant status and immunity. In: DIAZ DE (Ed): The Mycotoxin Blue Book, Nottingham University Press, Nottingham, pp. 93-138

ZIPRIN RL, MCMURRAY DN 1989: Influence of T-2 toxin on BCG vaccine efficacy against murine pulmonary tuberculosis. Vet Hum Toxicol **31**: 213-217

ZIPRIN RL, ELISSALDE MH 1990: Effect of T-2 toxin on resistance to systemic *Salmonella typhimurium* infection of newly hatched chickens. Am J Vet Res **51**: 1869-1872

ACTA VET. BRNO 2006, 75: 393-401; doi:10.2754/avb200675030393

Morphological Image of Fresh and Cryopreserved Dog Semen Evaluted by the Strict Analysis of Sperm Morphology Method, Using Sperm Quality Analyzer (SQA IIc) Evaluation

P. PŘINOSILOVÁ[1], A. VINKLER[2], Z. VĚŽNÍK[1]

[1]Veterinary Research Institute, Brno, Czech Republic
[2]University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic

Received October 4, 2005
Accepted April 13, 2006

Abstract

Přinosilová P., A. Vinkler, Z. Věžník: Morphological Image of Fresh and Cryopreserved Dog Semen Evaluated by the Strict Analysis of Sperm Morphology Method, Using Sperm Quality Analyzer *(SQA IIc)* Evaluation. Acta Vet Brno 75, 2006: 393-401.

Thirty fresh ejaculates from 15 dogs were cryopreserved in Tris-fructose-citric acid-egg-yolk extender with a glycerol content of 6%. Semen samples were examined by the methods of routine sperm analysis and by the SQA IIc device. The routine semen examination focused on the evaluation of parameters determining the quality of sperm membranes. The significance of monitoring semen quality in the course of the short-term survival test for predicting dog semen quality after thawing was assessed. Relevance of the assessment of sperm morphology, and above all the percentage of sperm with membrane changes in the acrosomal region was documented. The fact that the SQA device analyses semen quality by evaluating the mass of moving cells was confirmed. The results provided by the SQA IIc device appear insufficient for the needs of deeper dog semen analysis, especially morphology assessment.

Dog, sperm analysis, morphological assessment, Sperm Quality Analyser (SQA)

The testicles of breeding males are marked by their high cell production, accompanied by the natural production of insufficiently high-quality cells. A series of factors in the internal and external environment influence the production of this cell population. The proportion of abnormal cells in semen is linked to its fertilisation capacity. Morphological abnormalities of sperm in different animal species have been evaluated in connection with declines in fertility by a number of authors (Hancock 1959; Held et al. 1991; Oettlé 1993) . The limit values for the occurrence of morphologically abnormal sperm in semen have been established according to convention in different species of livestock, or in the human being, and are variable. Johnston et al. (2001) and Stockner and Bardwick (1991) set the percentage of morphologically abnormal sperm in normal dog ejaculate at below 20%. Threlfall (2003) considers acceptable a semen sample containing more than 70% morphologically normal sperm. Věžník et al. (2003) agreed with this value, with the proviso that primary defects in the ejaculate should not exceed 10%. According to Oettlé (1993) the fertility of fresh dog semen is markedly reduced when the percentage of morphologically abnormal cells in the semen is higher than 40%. For semen samples with a morphologically abnormal sperm below 40% the author obtained a pregnancy rate of 61%; when morphological defects exceeded 40%, this rate fell to 13%. Cryopreservation intervention reduces dog ejaculate quality and thus also the fertilisation success rate (Johnston et al. 2001). Feldman and Nelson (1987) consider fresh dog semen containing more than 70% of normal sperm and less than 20% of sperm with primary defects suitable for cryopreservation.

It is essential for the AI doses that donor semen quality is good because fertility of frozen dog semen, in additon to other factors, is associated with the quality of the fresh ejaculate

Address for correspondence:
MVDr. Petra Přinosilová
Veterinary Research Institute
Hudcova 70, 621 32, Brno
Czech Rupublic

Phone: +420 533 331 434
Fax: +420 541 211 229
E-mail: prinosilova@vri.cz
http://www.vfu.cz/acta-vet/actavet.htm

after collection (Peňa et al. 1999). Nevertheless, the quality of sperm of some dogs declines more after cryopreservation than sperm of other dogs. This may be caused by variations in the quality of cell membranes (Eilts 2005). Due to the fact that resistance of dog spermatozoa to the process of cryopreservation is low (Tsutsui et al. 2000) it is important to monitor the integrity of the sperm plasma membrane when performing resistance tests in fresh semen. Acrosomal membranes are pre-programmed for membrane fusion. Accordingly, they are the most labile of all sperm membranes (Graham 2001). Changes in the character of membrane swelling and loosening or loss of acrosome are evaluated during the assessment of morphological characteristics of sperm acrosomal membrane integrity. Foote (1975) views morphological assessment of acrosomal integrity as the most reliable method for prediction of semen fertility.

The morphological examination of semen constitutes one of the most objective methods of assessment and great attention has been devoted to it in a number of studies (Bartlett 1962; Check et al. 1992; Oettlé 1993; Menkveld and Kruger 1995; Root Kustritz et al. 1998; Kuster et al. 2004). The first description of dog sperm was published by Leeuwenhoek in 1679 (Oettlé 1993). One of the first to characterise and classify morphologically abnormal sperm was Lagerlöf (1936). This classic form of evaluation was also used with small variations by Bretschneider (1948), Bonadona (1956) and a number of others. Some studies place most importance on establishing the time, species and site of noxious substance on the characteristics of changes (Menger and Menger 1981). The development of the so-called strict morphological analyses (Kruger et al. 1986; Menkveld and Kruger 1995) allowed expanded and more accurate morphological diagnosis. For classification, the entire spermatozoon is taken into account and emphasis is placed on the multiparametric examination of spermatozoa, i.e. all changes present in the spermatozoon are evaluated. Any deviations from normal shape and structure are considered abnormal. Relating abnormalities to individual parts of the spermatozoon and dividing them into primary (developmental, major) and secondary (obtained, minor) enables us to better orientate ourselves in determining the place and time of the emergence of the abnormalities. This separation was noted while developing the computer programme SASMO (Strict Analysis of Sperm Morphology, Věžník et al. 2001).

The SQA IIc was developed for human semen analysis. The device assesses the quality of semen by creating the SMI (sperm motility index) parameter. Parameters of the device designated as sperm concentration, percentage of motile spermatozoa and percentage of sperm with normal morphology are derived by the device from SMI values on the basis of a conversion by means of specific algorithms (Comodo et al. 1997). The relationship between dog sperm motility and concentration and the SMI values has been studied by some authors (Iguer-Ouada and Verstegen 2001), but the relationship between percentages of morphologically aberrant sperm relative to the SMI values has not yet been investigated.

The aim of our research was to show the importance of morphological examination, focusing on establishing the secondary sperm abnormalities characterised as damage to the acrosome. We tested the hypothesis that it is important to monitor the parameters of dog sperm analysis in the course of the short-term survival test in order to predict semen quality after thawing.

We have also verified the possibility of using the SQA IIc device for detailed dog semen analysis. Most importantly, usability of the SQA IIc device for the assessment of dog semen morphology was investigated.

Materials and Methods

The animals and the methodology for collecting semen

Fifteen dogs of different ages from 2 to 11 years (average age 5) were included in the experiment, from the following breeds: Afghan Hound, Basset, Beagle, Doberman, Yorkshire Terrier, Labrador, German Shepherd,

Rottweiler, Leonberger, Shetland Sheepdog and Irish Wolfhound. The dogs were owned by private keepers who brought them to the Clinic of diseases of dogs and cats for experimental semen collection and examination. The semen was collected repeatedly by manual manipulation into plastic test-tubes with a funnel, without presence of a bitch. Clenching the collector in the palm sufficed to minimize the thermal shock. The second sperm rich fraction of each semen sample was collected. In total, 30 fresh semen samples were included for examination.

Examining the semen

After collection, the sperm concentration, percentage of motile and live sperm and the morphological image were established. The concentration was examined haemocytometrically in a Bürker Chamber, the motility was examined using the counting method in a microscope under 200 - 400 × magnification. A drop of semen was placed on a microscope slide and covered with a cover-slip, at 35 ºC. Eosin-nigrosin stain was used for distinguishing live from dead sperm. The morphological assessment was carried out by monitoring the samples stained according to Karras (Gamčík et al. 1976). The samples were examined under an optical microscope at magnification 1000 × using oil immersion, and were evaluated using the multiparametric method of evaluation with the SASMO (Strict Analysis of Sperm Morphology) programme (Věžník et al. 2001). The evaluation concentrated on the percentage of morphologically abnormal sperm, the percentage of sperm with primary defects, the percentage of secondary acrosomal defects and the percentage of secondary defects of tail.

The parameters of the SQA IIc device (SQA IIc, Medical Electronic Systems LTD, San Diego, CA, USA) were set as follows: SMI (sperm motility index), sperm motility, sperm concentration and normospermia. The last three indicators have been named "WHO parameters" by the device manufacturer, because they are listed in the WHO manual as criteria for the qualitative assessment of semen (WHO 1999).

The functional, morphological and SQA parameters were also established after the conclusion of the 120 min survival test. The test was carried out at laboratory temperature (22 °C) in semen diluted with physiological saline buffered with phosphate at a pH of 7.2. This pH was selected because of its activating effect on sperm motility. The ejaculates were diluted at a concentration of 100×10^6 spermatozoa/ml. After 120 min, the semen samples were heated to 35 °C and then evaluated.

Long-term cryopreservation and thawing of semen

Individual ejaculates were centrifuged at 500 *g* for 10 min; semen plasma was separated and then frozen and stored at -18 °C. The semen was diluted with Tris-fructose-citric acid-egg-yolk extender (modified according to Andersen 1975) with a glycerol content of 6% at a final concentration of $250 - 750 \times 10^6$ spermatozoa/ml. The diluted semen was poured into 0.5 ml labelled straws and the ends were sealed with polyvinylalcohol. The straws were equilibrated at 4 °C for 2 h and then frozen in liquid nitrogen steam in a metal straw-holder frozen to -100 °C. After 10 min they were stored in liquid nitrogen.

After thawing in a water bath at a temperature of 65 °C for 6 s, the semen samples were diluted to an approximate concentration of 100×10^6 spermatozoa/ml in physiological saline buffered with phosphate to a pH of 7.2 at 35 °C, and containing 20% autologous semen plasma. The parameters of sperm analysis described above were again established.

The quality evaluation of fresh ejaculates was performed by comparing the obtained values with the conventional limits of currently accepted criteria for fresh dog ejaculate quality (Věžník et al. 2000). These authors suggest that the values of evaluated parameters should not decrease by more than 10% from those of fresh semen during the short-time survival test. The parameters of semen samples after thawing were compared with the limit values of thawed dog semen samples based on the results of Linde-Forsberg and Forsberg (1989).

The relationships of the semen quality parameters were evaluated by statistically assessing changes during the survival test and during cryopreservation. Changes in the parameter values of sperm analysis during cryopreservation were established as a percentage of the increase or decline in values after thawing compared with the initial values of the fresh semen after collection. Similarly, the changes in values during the survival test were established by comparing the values obtained after 120 min with the initial values after collection. The parameter values for routine sperm analysis were statistically compared with the parameters of the SQA IIc device.

Statistical methods

Statistical analysis was performed using the Stat Plus software (Stat Plus, version 1.01, 1991, VRI Brno). The Pearson Correlation test was used for evaluation of correlations for parametric data. The Spearman test was used for evaluation of correlations for non-parametric data. Data were evaluated for differences between groups using the Paired t-test for parametric data and Wilcoxon Signed Rank Test for non-parametric data. The level of significance was set at $p < 0.05$. The Kruskal-Wallis Test was used for analysis of variances.

Results

Tables 1 and 2 show the average values for the parameters monitored of fresh and thawed semen.

The lower limits of the qualitative criteria and the proportion of ejaculates corresponding to the conventional values in the given indicators are shown in Table 3. Table 4 shows comparison of the values of assessed parameters with the limit values that determine the quality of thawed dog semen.

Table 1. Average values of parameters of the routine analysis of semen during the course of the short-term survival test and after thawing (n = 30)

		Concentration	Motility	Live sperm	Morphologically abnormal sperm	Normospermia	Sperm with primary defects	Sperm with secondary defects of acrosome	Sperm with secondary defects of tail
		$\times 10^6$/ml	%	%	%	%	%	%	%
1	Average±SD	276.3±233.51	78.0±11.79	83.8±10.23	27.0±12.52	73.0±12.52	10.5±5.85	8.5±8.28	8.7±5.39
	Max	1225.6	95.0	96.2	53.5	92.0	28.5	34.0	25.0
	Min	51.2	55.0	56.9	8.0	46.5	3.5	1.0	2.5
2	Average±SD	nd	52.3±18.70	75.8±11.13	36.1±17.98	63.9±17.98	10.3±6.81	15.42±15.14	13.4±10.78
	Max	nd	90.0	91.8	82.6	87.0	35.5	65.2	49.8
	Min	nd	10.0	48.3	13.0	17.4	2.0	3.0	3.0
3	Average±SD	91.4±28.72*	41.5±12.74	55.5±10.75	57.5±12.92	42.5±12.92	9.1±4.89	41.3±11.34	18.7±8.90
	Max	150.4	70.0	88.2	78.5	67.5	19.5	68.5	38.0
	Min	49.6	5.0	38.0	32.5	21.5	1.5	21.0	2.0

1 initial values of fresh semen
2 values after 120-min survival test of fresh semen
3 values of the parameters of semen after thawing
SD standard deviation
* sperm concentration after diluting thawed sample at an assumed concentration of 100×10^6 spermatozoa /ml
nd not determined

Table 3. Proportion of ejaculates corresponding to conventional limits accepted for the qualitative evaluation of dog semen during the survival test (n = 30)

Parameter		Motility		Live sperm		Morphologically abnormal sperm	
Time after collection		0 min	120 min	0 min	120 min	0 min	120 min
Conventional limits for the parameters (%)		>70	>63	>70	>63	<30	<36
Proportion of ejaculates corresponding to the conventional limits (%)	established by routine sperm analysis	76.7	26.7	90.0	86.7	66.7	66.7
	established by the SQA device	96.7	56.7	nd	nd	0	0

nd not determined

The relationship between the values of examined parameters obtained during cryopreservation and the values obtained during the survival test was investigated. A highly significant correlation was found between the decline in the live sperm percentage during the survival test of fresh semen and the decline of this parameter values during cryopreservation ($r = 0.519, p < 0.01$).

There was a statistically non-significant correlation between the increase of secondary acrosomal defects during the survival test of fresh semen and the increase of these changes during cryopreservation. However, regression analysis showed an obvious positive trend. A statistically significant negative correlation ($r = -0.441, p < 0.05$) was found between the percentages of sperm with acrosomes showing membrane changes after the 120 min survival test and percentages of live sperm after thawing. Different levels of disturbed membrane integrity in the area of the acrosome as assessed by the viability test using eosin-nigrosin stain are shown in Fig. 1, 2 and 3 (see Plate II and III).

Table 2. Average values of SQA parameters of semen monitored in the course of the short-term survival test and after thawing (n = 30)

		Concentration	Motility	Normospermia	SMI
		$\times 10^6$/ml	%	%	%
1	Average±SD	203.1±24.24	84.7±6.10	53.5±3.05	520.1±62.91
	Max	225.0	91.0	57.0	582.0
	Min	107.0	62.0	43.0	293.0
2	Average±SD	nd	64.3±19.20	42.5±11.46	334.8±153.20
	Max	nd	89.0	56.0	564.0
	Min	nd	17.0	17.0	45.0
3	Average±SD	97.2±49.93*	54.7±18.06	36.6±11.85	253.3±119.74
	Max	183.0	79.0	51.0	458.0
	Min	0.0	0.0	0.0	26.0

1 initial value of fresh semen
2 value after 120-minute survival test of fresh semen
3 value of the parameters of semen after thawing
SD standard deviation
SMI sperm motility index
* sperm concentration after diluting thawed sample at an assumed concentration of 100×10^6 spermatozoa /ml
nd not determined

Table 4. Proportion of semen samples corresponding to the limits selected by our laboratory for the use in dog semen after thawing (n = 30)

Parameter		Motility	Live sperm	Morphologically abnormal sperm
Chosen limits for the parameters (%)		>40	>40	>60
Proportion of ejaculates corresponding to the limits (%)	established by routine sperm analysis	73.3	96.7	60.0
	established by SQA device	80.0	nd	53.3

nd not determined

The relationship between the decline of fresh sperm motility during the survival test and the decline of this parameter values during cryopreservation, and the relationship between the increase of morphologically abnormal sperm during the survival test and their increase during cryopreservation was statistically non-significant.

Table 5. Correlations between the values of routine analysis of semen and of the SQA device and the statistical significance of the differences in the averages of these values (n = 30)

		Motility vs. motility (SQA).	Concentration vs concentration (SQA)	Normospermia vs. normospermia (SQA)	Morphologically abnormal sperm vs. SMI
1	*r*	0.239[b]	0.570[a]	0.260[b]	-0.261[b]
	t-test	a	b	a	nd
2	*r*	0.792[a]	nd	0.241[b]	-0.223[b]
	t-test	a	nd	a	nd
3	*r*	0.666[a]	0.215[b]	0.098[b]	-0.072[b]
	t-test	a	b	b	nd

1 initial value of fresh semen
2 value after 120-minute survival test
3 value after thawing
SMI sperm motility index
a statistical significance where $p < 0.01$
b statistically nosignificant
nd not determined
r correlation coefficient

Analysis of variance between the values of sperm with primary defects after collection and the values after the 120 min survival test and after thawing was carried out in order to verify the diagnostic relevance of morphological examination. Statistical non-significance of variances demonstrated concordance in these findings.

The statistically significant correlation between the increase in the percentages of morphologically abnormal sperm and sperm with secondary acrosomal defects during the survival test ($r = 0.508$, $p < 0.01$) and also during cryopreservation ($r = 0.843$, $p < 0.01$) was detected.

The correlations between values of determined parameters established by routine sperm analysis and the SQA device and the differences between the groups are shown in Table 5. Regression analysis of the relationship between routinely determined sperm concentrations and those determined by the SQA device showed a logarithmic character of correlation at the initial value.

Discussion

The integrity and normal resistance of the plasma membrane of sperm is a necessary prerequisite of successful fertilization, as only sperm with intact plasma membranes are capable of fertilization (Peňa et al. 1999). The importance of the sperm membrane quality assessment during the survival test for the prediction of semen quality after thawing has been documented by statistical evaluation of the results. This is consistent with the results obtained by Věžník et al. (1986) who confirmed that the increase in secondary alterations in bull sperm during the 120 min survival test significantly correlated with sperm quality after thawing (freezability).

The assessment of the percentage of sperm with secondary changes reveals sperm with disturbed membrane integrity in the area of the acrosome, but perhaps not so substantially disturbed that it manifests during the viability test with the eosin-nigrosin stain. On the other hand, a number of sperm shown by intravital dye to be dead need not have a microscopically disturbed membrane in the area of the acrosome, and therefore both parameters of investigation are important. Different levels of disturbed membrane integrity in the area of the acrosome assessed by the viability test with eosin-nigrosin stain are shown in Fig. 1, 2 and 3. Sperm with moderately disturbed membrane integrity in the area of the

acrosome may even be motile, as long as they are not also damaged in the tail area. Nevertheless from the point of view of their future success in the fertilisation process, such sperm are not promising because their life is limited. This conclusion corresponds to Oettlé's (1986) assertion concerning the inadvisability of evaluating semen quality solely on the basis of motility itself, because motile sperm need not always be fertile.

The higher occurrence of the secondary acrosomal defects may be partly due to methods employing the morphological dyes. Stains emphasising the structures of the acrosome reveal more damaged cells than overall methods of staining. As Root Kustritz et al. (1998) and Root and Johnston (1994) state, methods of staining and the preparation technique also have an influence on the results of the morphological assessment. Any staining method causes the occurrence of artefacts and may mask genuine defects. It is more difficult to assess stained samples of thawed semen than samples of fresh semen because elements of the extender, like egg-yolk or milk, may interfere with most stains (Oettlé 1986).

The results of our evaluation demonstrate the dependence of the increase in the proportion of morphologically abnormal sperm during storage or cryopreservation predominantly on the growth of secondary acrosomal changes, which corresponds to the assertion of Věžník et al. (2005). The lower percentage of ejaculates corresponding to the established limits for percentage of sperm with morphological defects in fresh and thawed samples (Table 3, 4) demonstrated the relevance of sperm morphology assessment in addition to the parameters of motility and percentage of live sperm. It follows from the present results that morphology of live or even motile spermatozoa may be abnormal.

The statistical non-significance of the correlation between the values of the decline in motility during the survival test and during cryopreservation is caused by the high drop in sperm motility during the 120 min survival test. This marked sperm motility decrease may be the result of using a physiological saline buffered to a pH of 7.2 as a dilution medium. This pH, chosen to activate sperm motility, may be less suitable for certain ejaculates because of its high alkalinity, which by supporting higher movement activity results in faster exhaustion. It is possible to explain the non-significance of the correlation between the increase of morphologically abnormal sperm during cryopreservation and the increase of these changes during the short-term survival test by the differential occurrence of secondary changes to the tail after collection, during the 120 min survival test and after thawing. The highest occurrence and also increase in secondarily changed tails in comparison with the values after collection was found in semen after thawing.

The SQA device analyses semen quality by evaluating the mass of moving cells i.e. it assesses a semen sample on the basis of two parameters: motility and concentration. By evaluation of the results obtained by the SQA, the relationships between concentration and motility of spermatozoa were demonstrated. In cases of marked differences between concentration and motility, distorted SMI values were found. For this reason, a mutual correlation between the motility values established by the classic counting method and using the SQA in the whole set of ejaculates at the initial value after collection was not attained. After eliminating semen samples with extreme differences between concentration (higher than 200×10^6 spermatozoa/ml) and motility (lower than 70%), a correction of the relations occurred and the motility values established by the SQA correlated with the motility established by counting ($r = 0.679$; $p < 0.01$; n = 20). Also, the differences in means were statistically non-significant. Regression analysis of the initial concentration values established routinely and by the SQA device showed a logarithmic character of the relationship. This is influenced by the maximum concentration values that can be evaluated by SQA. The maximum possible concentration given by the device was 225×10^6 spermatozoa/ml. Iguer-Ouada and Verstegen (2001) did not observe

a statistically significant correlation over the tested 200×10^6 cells/ml, possibly because of a saturation of the system.

A semen sample with very low sperm motility is given a low SMI index by the device, but sets zeroes for the so-called WHO parameters. The results recalculated from SMI using the device algorithms and designated as sperm motility and normospermia have been modulated according to the limit values. Therefore, the percentage of motility established by the SQA device ranged from zero to 91%. The quantity of normal cells is set from zero to 57%, given the maximum SMI value is obtained. These restricted values of normospermia assessed by SQA device caused that the obtained ejaculates were not of acceptable quality according to the mentioned conventional quality limits (Table 3).

Conclusion

The hypothesis that it is important to monitor the parameters of sperm analysis during the survival test in order to predict semen quality after thawing was tested.

The relevance of evaluation of sperm morphology, especially the percentage of sperm with membrane changes in the acrosomal area, was documented.

While using the SQA IIc device for evaluating the quality of dog semen samples, it was confirmed that the SQA device analyses ejaculate quality by evaluating the mass of moving cells. The results provided by the SQA IIc device appear insufficient for the needs of deeper dog semen analysis, especially morphology assessment.

Morfologický obraz nativního a konzervovaného semene psa a jeho hodnocení metodou Striktní analýzy morfologie spermií, s využitím hodnocení přístrojem Sperm Quality Analyzer (SQA IIc)

30 nativních ejakulátů od 15 psů bylo konzervováno Tris-fruktoso-citrát-žloutkovým ředidlem s obsahem glycerolu 6 %. Semeno bylo vyšetřeno metodami rutinní spermatoanalýzy a přístrojem SQA IIc. Rutinní vyšetření semene bylo zaměřeno na hodnocení parametrů určující kvalitu membrán spermie.

Byl ověřen význam sledování parametrů spermatoanalýzy v průběhu krátkodobého testu přežitelnosti pro predikci kvality semene psa po rozmrazení. Byla doložena významnost posouzení morfologického obrazu semene, a to především zastoupení spermií s membránovými změnami v oblasti akrosomu. Byla potvrzena skutečnost, že přístroj SQA analyzuje kvalitu ejakulátu na základě zhodnocení masy pohybujících se buněk. Pro potřeby hlubší spermatologické diagnostiky semene psa, zvláště pak zaměřené na morfologickou analýzu semene, se jeví výsledky poskytnuté přístrojem SQA IIc jako nedostačující.

Acknowledgement

This work was supported by associated resources for research work: The Fund for University Development Project No. 1271/2001, VZ MSM 6215712403 and the Ministry of Agriculture Project No. 0002716201.

References

ANDERSEN K 1975: Insemination with frozen dog semen based on a new insemination technique. Zuchthygiene **10:** 1

BARTLETT DJ 1962: Studies on dog semen. I. Morphological characteristics. J Reprod Fertil **3**: 173-189

BONADONA T 1956: Di una particolare formazione alla testa dei nemaspermi bovini mesa in evidenze al microscopia elettronico. Riv Biol **47**: 297

BRETSCHNEIDER LH, VAN ITERSON W 1948: Het electronenmicroscopisch Onderzoek van het Spermium van de Stier. Tijdschr Dierg **73**: 233

CHECK JH, ADELSON HG, SCHUBERT BR, BOLLENDORF A 1992: Evaluation of sperm morphology using Kruger's strict criteria. Arch Androl **28**: 15-17

COMODO F, INAUDI P, PETRILLI S, D'ANTONA N 1997: Sperm quality analyzer (SQA): A reliable tool for semen parameters evaluation. In: Proc. 10th World Congress for In Vitro Fertilization and Assisted Reproduction. Vancouver, Canada, 24 p.

EILTS BE 2005: Theoretical aspects of canine semen cryopreservation. Theriogenology **64**: 692-697

FELDMAN EC, NELSON RW 1987: Infertility of the bitch and associated breeding disorders. In: FELDMAN EC, NELSON RW: Canine and Feline Endocrinology and Reproduction. W.B. Saunders, Philadelphia, pp. 454-480.

FOOTE RH 1975: Semen quality from the bull to the freezer: an assessment. Theriogenology **3**: 219-234

GAMČÍK P, KOZUMPLÍK J, SCHWARC F, VLČEK Z, ZIBRÍN M 1976: Artificial insemination and andrology in livestock (in Czech). Príroda, Bratislava. 574 p.

GRAHAM JK 2001: Assessment of Sperm Quality. AAEP Proceedings **47**: 302-305

HANCOCK JL 1959: The morphologic characteristics of spermatozoa and fertility. Int J Fertil **4**: 347-359

HELD JP, PRATER P, STETTLER M 1991: Spermatozoal head defect as a cause of infertility in a stallion. J Am Vet Med Assoc **199**: 1760-1761

IGUER-OUADA M, VERSTEGEN JP 2001: Validation of the sperm quality analyzer (SQA) for dog sperm analysis. Theriogenology **55**: 1143-1158

JOHNSTON SD, ROOT KUSTRITZ MV, OLSON PNS 2001: Semen Collection, Evaluation, and Preservation. In: JOHNSTON SD, ROOT KUSTRITZ MV, OLSON PNS: Canine and Feline Theriogenology. W.B. Saunders, Philadelphia, pp. 287-306.

KRUGER TF, MENKVELD R, STANDER FSH, LOMBARD CJ, VAN DER MERVE JP, VAN ZYL JA, SMITH K 1986: Sperm morphologic features as a prognostic factor in vitro fertilization. Fertil Steril **46**: 1118-1123

KUSTER CE, SINGER RS, ALTHOUSE GC 2004: Determining sample size for the morphological assessment of sperm. Theriogenology **61**: 691-703

LAGERLÖF, N. 1936: Sterility of bulls. Vet Rec **12**: 121-129.

LINDE-FORSBERG C, FORSBERG M 1989: Fertility in dogs in relation to semen quality and the time and site of insemination with fresh and frozen semen. J Reprod Fertil **39** (Suppl): 299-310

MENGER H, MENGER S 1981: Comparative light and electron microscopic studies of spermiogenesis in the male rabbit, boar and ram. Arch Exp Vet Med **35**: 359-74

MENKVELD R, KRUGER TF 1995: Advantages of strict (Tygerberg) criteria for evaluation of sperm morphology. Int J Androl **2**: 36-42

OETTLÉ EE 1986: Changes in acrosome morphology during cooling and freezing of dog semen. Anim Reprod Sci **12**: 145-150

OETTLÉ EE 1993: Sperm morphology and fertility in the dog. J Reprod Fertil **47** (Suppl): 257-260

PEÑA A, JOHANNISSON A, LINDE-FORSBERG C 1999: Post-thaw evaluation of dog spermatozoa using new triple fluorescent staining and flow cytometry. Theriogenology **52**: 965-980

ROOT MV, JOHNSTON SD 1994: Basics for a Complete Reproductive Examination of the Male dog. Semin Vet Med Surg (Small Animal) **9**: 41-45

ROOT KUSTRITZ MV, OLSON PN, JOHNSTON SD, ROOT TK 1998: The Effects of Stains and Investigators on Assessment of Morphology of Canine Spermatozoa. J Am Anim Hosp Assoc **34**: 348- 352

STOCKNER PK, BARDWICK C 1991: The Relationship of Semen Parameters to Fertility in the Dog. Canine Pract **6**: 15-23

THRELFALL WR 2003: Semen Collection and Evaluation. In: ROOT KUSTRITZ MV: Small Animal Theriogenology. Butterworth Heinemann, Missouri, pp. 97-123.

TSUTSUI T, HASE M, HORI T, ITO T, KAWAKAMI E 2000: Effects of Orvus ES Paste on canine spermatozoal longevity after freezing and thawing. J Vet Med Sci **62**: 533-535

VĚŽNÍK Z, ŠVECOVÁ D, LÁNÍKOVÁ A, JURMANOVÁ K, VOJTÍŠEK B 1986: Hygienic aspects of fungal and other contaminations of the bull semen. Annual report of the phase P 06-329-809-01-04 (in Czech). Veterinary Research Institute, Brno, 63 p.

VĚŽNÍK Z et al. 2000: Evaluation of semen for assisted reproduction and selection of breeding males - Strict analysis of sperm morphology SASMO (in Czech). Veterinary Research Institute, Brno, 105 p.

VĚŽNÍK Z, MATOUŠKOVÁ O, ŠVECOVÁ D, ZAJÍCOVÁ A 2001: The use of the computer technology for the evaluation of the strict morphological sperm analysis. Vet Med-Czech **46**: 35-40

VĚŽNÍK Z, ŠVECOVÁ D, ZAJÍCOVÁ A, PŘINOSILOVÁ P 2003: Functional evaluation of dog ejaculates with a priority aspect of acrosome integrity. Vet Med-Czech **48**: 221-228

VĚŽNÍK Z et al 2005: Continuing professional education in spermatology and andrology and methods of sperm analysis (in Czech). Veterinary Research Institute, Brno, 197 p.

WORLD HEALTH ORGANIZATION 1999: WHO laboratory manual for the examination of human semen and sperm-cervical mucus interaction. 4th ed. The Press Syndicate of the University of Cambridge, Cambridge, 128 p.

ACTA VET. BRNO 2006, 75: 403-410; doi:10.2754/avb200675030403

Evaluation of Marginal Microgaps of Two Glass-ionomer Cements (GIC) in Dogs and Sheep *in vivo*

M. FIGUROVÁ, V. LEDECKÝ, S. ŠTVRTINA[1]

Clinic of Surgery, Orthopaedics and Roentgenology, University of Veterinary Medicine in Košice, Slovakia
[1]Medical Faculty of Komenský University in Bratislava, Slovakia

Received February 7, 2005
Accepted April 13, 2006

Abstract

Figurová, M., V. Ledecký, S. Štvrtina: Evaluation of Marginal Microgaps of Two Glass-ionomer Cements (GIC) in Dogs and Sheep *in vivo*. Acta Vet. Brno 2006, 75: 403-410.

The aim of the experiment was to evaluate the marginal microgaps of two ionomer cements: Kavitan Plus (Spofa Dental) and Vitremer (3M ESPE) in dog and sheep dentition *in vivo*.

Dentitions of sheep and dogs were restored *in vivo* with a conventional, glass polyalkenoic, chemically activated cement Kavitan Plus with hydrophilic properties capable and with a resin-modified glass-ionomer cement Vitremer with light-induced polymerization and auto-polymerization reaction of methyl metacrylate group.

The parameters of glass-ionomers were evaluated in 6 groups of animals, 2 animals in each, at various time intervals (after 1, 4 and 6 months in dogs and 3, 6 and 9 months in sheep, starting from the beginning of the experiment). The restorative materials were placed to buccal surfaces of permanent teeth. At the intervals specified, under general injection anaesthesia, throughout the experiment we extracted 24 teeth from sheep and 30 from dogs. When processing the samples of dog's teeth two samples were damaged. One month after the placement, Kavitan plus restorations became loose only in one case in dogs (80% successfulness). In sheep two Kavitan Plus restorations became loose after 9 months (50% successfulness). During the experiment we observed neither cracks nor marginal discoloration in both Kavitan Plus and Vitremer restorations. Statistically significant ($P = 0.04$) differences were observed in the dentin of dogs receiving glass-ionomer Vitremer restorations which exhibited lower marginal microgaps. The remaining results were non- significant (ANOVA test). Fluoride ions released from GIC support the treatment of dental hard tissues. These materials could be used as definitive restorations of class A - D cavities in dogs and dental cervical caries in sheep as well as underlying layers of composite and amalgam materials.

Glass-ionomer cement, experiment, dentition, incisors, premolars, histology, microgaps, restoration

Veterinary stomatology faces high prevalence of periodontitis. The second most frequent stomatologic diagnosis in carnivores are various types of teeth fractures with or without open pulp cavities the therapy (Capík 2005; Nemec et al. 2005). Less frequent are dental caries, luxation of teeth, enamel hypoplasia and tumours.

The treatment of enamel hypoplasia disease is based on stomatological restorations. The use of restorations in the treatment of various degrees of enamel hypoplasia was described by Capík (1996). Due to limited possibility of cavity preparation and intensive masticatory forces in dogs (about 10 times stronger than in humans) the lifetime of restorations of extensive defects in the coronal third of the crown is much shorter compared to those in the apical part of the crown.

Caries dentini is a disease manifested by decay of dental hard tissues. Its development is conditional on the presence of caries-susceptible teeth, micro-organisms and food. The overall incidence of caries in dogs ranges between 5-35%. The most frequently affected teeth are first maxillary molars and first and second mandibular molars (Capík et al. 1999). The low incidence of caries in dogs has been attributed particularly to increased neutralising and antibacterial properties of dog saliva.

Address for correspondence:
MVDr. Mária Figurová
Department of Surgery
University of Veterinary Medicine
Komenského 73, 041 81 Košice, Slovak Republic

Phone: +421 556 337 832
Fax: +421 556 337 832
E-mail: composite@orangemail.sk
http://www.vfu.cz/acta-vet/actavet.htm

Hale (1998) recorded a 40% incidence of caries in pits and fissures of canine teeth, 36% incidence of surface crown caries, and 23% incidence of root caries. Because of considerably different anatomy of human and animal teeth the Black's system of cavity description has not been generally accepted. With carnivores a system developed by Harvey and Emily has been used which divides the dental damage and caries into 6 classes, A, B, C, D, E, F, according to the location (Harvey and Emily 1993).

In the stomatological practice we use different types of restorations. Glass-ionomer cements (GIC) are restorative materials of a new generation which, with regard to their hydrophilic nature, adherence to hard dental tissues and gradual release of fluoride ions, became important restorative materials for dental cervical caries and lesions (Glasspoole and Erickson 1993). GICs are composed of a basic glass and an acidic polymer. The acid-base reaction of these two components is typical only of this type of restorative materials (McLean et al. 1994). Enrichment of GIC with resins (hybrid, resin-modified GIC) or metals (metal ionomers) ensures better physical properties of restorations compared to conventional poly(alkenoic) GIC.

The mechanism of chemical adhesion between GIC and dental hard tissues ensures minimum to nil microleakage (Attin at al. 1995) and minimises the quantity of dentin that has to be removed by drilling during the preparation.

Marginal leakage may result in various pulp pathologies, development of secondary caries and discoloration adjacent to restorations. The biggest problem concerning marginal adaptation is associated with the zone of cement/dentinal interface which is known for high humidity of the operative field. Negative factors related to polymerisation shrinkage and thermal expansion resulted in post-operative sensitivity of composites (Mitra and Conway 1994). GIC showed minimum variations of the coefficient of thermal expansion (Puckett et al. 1995).

Despite the shortcomings of GIC, such as low mechanical resistance, sensitivity to a very dry or very humid environment, these materials are justified in some situations in which composites or amalgams appear unsuitable. Owing to them a mineralisation mound develops which is registered in the process of reconstruction of impaired dentin (Khouw-Liu et al. 1999). Their anticariogenic effect has been confirmed also in the environment supporting the dental caries. The effect of acids on the surface of GIC was less pronounced in comparison with composites (Glasspoole and Erickson 1993; Park and Kim 1997; Donly and Grandgenett 1998).

A smear layer fills up the orifice of dentinal tubules. It contains small particles of collagen matrix, saliva, bacteria, tubular fluid and blood elements. The studies focusing on adhesion of GIC to hard dental tissues use two different procedures. These procedures are based on preservation or removal of the smear layer. Polyalkenoate GIC higher strength of GIC Fuji II LC after removal of the smear layer. The samples with GIC Fuji IX and Photac-Fil Quick, with and without a conditioner, showed no significant differences (Tanumiharja et al. 2000).

The aim of the experiment was to evaluate the marginal microgaps of two ionomer cements: Kavitan Plus (Spofa Dental) and Vitremer (3M ESPE) in dog and sheep dentition *in vivo*. Two animal species differring widely by the type of dentition, and processing of food were selected. These differences and the respective intraoral conditions may affect the restorations used in the experiment.

Materials and Methods

The experiment was carried out between June 2002 and May 2003 at Clinic of Surgery, Orthopaedics and Roentgenology, UVM Košice, accredited for experiments on animals conducted for scientific purposes (Act No. 115/95 of the Civil Code on Animal Protection), accreditation No. 12 766/02-220.

Two GIC were used to reconstruct class A cavities: Kavitan Plus (K), a chemically activated cement belonging to

the group of conventional glass polyalkenoic cements, and resin-modified Vitremer (V) which, besides light-induced polymerisation exhibits also autopolymerisation reaction of metacrylate group radicals without access of light. We evaluated the following parameters: marginal microgaps, colour stability, quality and retention of restorations of class A cavities *in vivo*. The experiment was conducted on animals with healthy dental tissues. The parameters of glass ionomers were evaluated in 6 groups of animals, 2 animals in each at various time intervals (at 1, 4 and 6 months in dogs and 3, 6 and 9 months in sheep after the beginning of the experiment). At the above-mentioned intervals, we extracted and evaluated 4 incisors and 4 premolars from sheep (a total of 24 teeth) and 6 incisors and 4 premolars from the dogs (a total of 30 teeth) under general injection anaesthesia. When processing the samples of canine teeth two samples were damaged. Histological and statistical data are therefore based on 28 teeth.

Preparation procedure

Calculus was cleaned off the teeth and isolated from the buccal mucosa by means of paper cylinders. The prepared class A cavities were on the buccal surface of teeth. Those located on the left side of dentitions were restored with GIC Kavitan Plus and those on the right side were restored with GIC Vitremer.

When using GIC Kavitan Plus we did not disturb the smear layer so we could observe the influence of this layer on the quality of restorations.

The powder was homogenised by shaking. The ratio of powder to liquid was 1:1. The procedure used complied with that recommended by the producer but the dentinal conditioner was omitted. After solidification, the excess material was removed and the restoration was coated with a protective glaze of LC Varnish.

Before application of Vitremer we used a primer which adjusted the smear layer. The procedure used corresponded fully to the manufacturers' instructions. After setting, the excess material was removed and the restoration was covered with a protective coat. Preparation of class A cavities and their restoration was carried out on animals under general anaesthesia. Premedication of all animals was achieved by administration of atropin a.u.v. at a dose of 0.05 $mg \cdot kg^{-1}$ i.m. The general anaesthesia in dogs was induced by i.m. administration of a combination of xylazin a.u.v. and ketamin a.u.v. at a dose of 1-2 $mg \cdot kg^{-1}$ and 5- 10 $mg \cdot kg^{-1}$, respectively. The general anaesthesia in sheep was induced by i.m. administration of a combination of xylazin a.u.v. and ketamin a.u.v. at a dose of 0.4 $mg \cdot kg^{-1}$ and 5-10 $mg \cdot kg^{-1}$, respectively. During the experiment the dogs were fed a combined diet consisting of dry granules and a soft component.

The preparation of histological specimens differed from the conventionally used procedure. Histological sections were not dehydrated. To ensure correct interpretation, care had to be taken to avoid undue dehydration of sections during processing and observation. The extracted teeth were conserved with 10% formalin. Specimens were prepared using water-hardened cyanoacrylates. Sections were cut with a diamond saw and after hardening were embedded in solacryl. They were prepared longitudinally and their thickness ranged between 100 and 150 μm (Plate III, Fig. 5, 6). The width of microgaps was measured in μm separately for the dentin and enamel. Statistical evaluation of the size of microgaps was carried out by ANOVA.

Results

Clinical evaluation

Sheep

All restorations in 3 groups (each n = 2) were intact and showed no marginal discoloration. In Group 3 (n = 2) of animals (9 months after the beginning of the experiment) Two restorations with GIC Kavitan Plus showed signs of separation (50% successfulness). Neither cracks nor marginal discoloration was observed in the remaining restorations (Table 1). No health complications occurred in the animals throughout the experiment and their food intake and digestion corresponded to their physiology.

Table 1. Retention and colour stability of GIC in sheep dentition

Sheep	Material	Number of evaluated teeth	Number of teeth with separation of restorations	Retention of restorations %	Colour stability %
1st group (after 3 months)	Vitremer	4	-	100	100
	Kavitan Plus	4	-	100	100
2nd group (after 6 months)	Vitremer	4	-	100	100
	Kavitan Plus	4	-	100	100
3rd group (after 9 months)	Vitremer	4	-	100	100
	Kavitan Plus	4	2	50	100

Dogs

Restorations in all groups (each n = 2) were intact and showed no discoloration.

In the 4th group of animals (dogs after 1 month of the experiment) 100% retention of all resin-modified GIC was observed. One chemically activated GIC restoration showed signs of separation i.e. successfulness of treatment was 80% (Table 2). The dogs showed no signs of health problems throughout the experiment their food intake and digestion was physiological.

Table 2. Retention and colour stability of GIC in canine dentition

Sheep	Material	Number of evaluated teeth	Number of teeth with separation of restorations	Retention of restorations %	Colour stability %
4th group (after 1 month)	Vitremer	5	-	100	100
	Kavitan Plus	5	1	80	100
5th group (after 4 months)	Vitremer	5	-	100	100
	Kavitan Plus	4*	-	100	100
6th group (after 6 months)	Vitremer	4*	-	100	100
	Kavitan Plus	5	-	100	100

*One sample was damaged during processing

Histology

Methods used: 3-factorial dispersion analysis of repeated measurements (method, tooth, measurement) was performed.

The measurements in sheep showed that the mean span of microgaps between dentin and Kavitan Plus GIC was 15.6 μm and Vitremer GIC restorations was 15.0 μm, resp. Current effect: F (1, 4) = 0.03, probability P = 0.87 (Fig. 1). As P was higher than 0.05, the nil hypothesis which claims that the means at the 5% level of significance are equal could not be rejected.

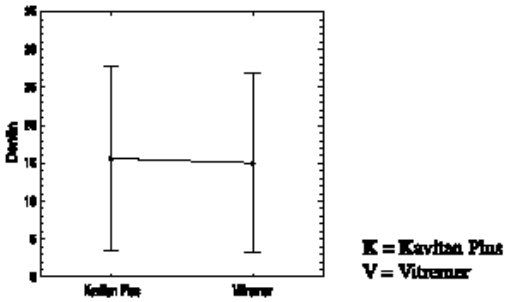

Fig. 1. Evaluation of marginal leakage in μm at sheep dentin

K = Kavitan Plus, V = Vitremer
Mean K = 15.6 μm V = 15.0 μm. Statistical significance of differences between means was determined by F-statistics.

Significant differences ($P = 0.04$) between the span of microgaps with the two restoration materials used were observed in dogs as the respective mean spans were 16.2 μm and 12.6 ûm with Kavitan Plus and Vitremer GIC, resp. Current effect: F (1, 6) = 6.70, $P = 0.04$, statistically significant (Fig. 2).

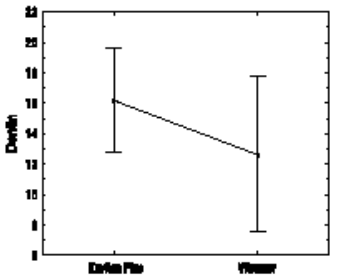

Fig. 2. Evaluation of marginal leakage in μm at dog dentin

Mean K = 16.2 μm, V = 12.6 μm
Current effect: F (1, 6) = 6.70, $P = 0.04$, statistically significant.

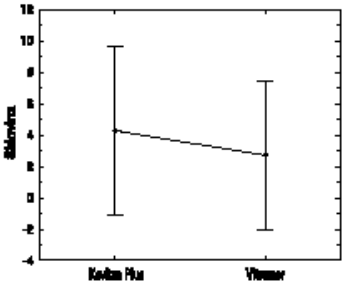

Fig. 3. Evaluation of marginal leakage in μm at sheep enamel

Mean K = 4.3 μm , V = 2.7 μm
Current effect: F (1, 5) = 0.54, $P = 0.50$, non-significant

The microgaps between sheep's enamel and Kavitan Plus GIC was 4.3 μm and Vitremer GIC restorations was 2.7 μm. Current effect: F (1, 5) = 0.54, $P = 0.50$, NS (Fig. 3).

Results of microgaps between dog's enamel and Kavitan Plus (2.15 μm) GIC and Vitremer GIC (2.85 μm) restorations were non-significant. Current effect: F (1,6) = 0.10, $P = 0.80$, NS (Fig. 4).

The span of microgaps at dentin, enamel and both GIC measured in our study had no effect on the development of pathological changes in dental tissues and marginal discoloration.

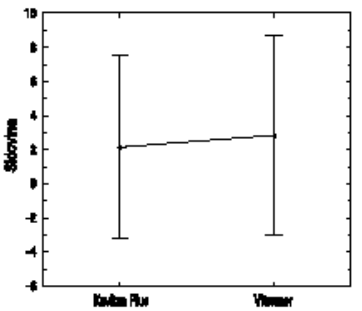

Fig. 4. Evaluation of marginal leakage in μm at dog enamel

Mean K = 2.15 μm , V = 2.85 μm
Current effect: F (1, 6) = 0.10, $P = 0.80$, NS

Discussion

The study describes real application of GIC in stomatological practice. Owing to their hydroscopic properties, GIC produce tight bond with dental hard tissues within the first 24 hours (Attin et al. 1995). This high marginal adaptation prevents ingress of fluids and bacteria

into the tooth which results in colour stability of restorations and decreased irritation of the pulp.

With regard to the release of fluoride ions and thermal expansion of GIC which resembles that of dentin and enamel, GIC have been advocated for the restoration of dental cervical lesions and caries (Abdalla and Alhadainy 1997; Harvey and Emily 1993). The results obtained in our study proved that better bonding of GIC was obtained with enamel apatite than with dentinal collagen fibres. Our measurements showed that the mean span of microgaps at enamel was between 2.15 and 4.3 μm whereas that at dentin ranged between 12.6 and 16.2 μm. Rigsby et al. (1990) used various dentin bonds (in composites) for cervical lesions and reported that the size of microgaps was between 5.5 and 18 μm. A two year study of GIC and compomer showed 90 - 100% successfulness with regard to retention and marginal adaptation of restorations. Compared to GIC, the compomer showed better colour stability of restorations as the reported colour stability of GIC was only 67 - 85% (Abdalla and Alhadainy 1997). Our study showed that after 9 months of the experiment in sheep and 6 months in dogs no marginal discoloration of restorations with both materials used was observed.

The studies of Najar et al. (2003) did not show increased adhesion of some GIC to dental hard tissues after removal of the smear layer. In vitro studies on bovine incisors point to increased adhesion of resin-modified GIC and compomers to conditioner-adjusted dentin (Buchalla et al. 1996). Better adhesion of experimental GIC to dentin of bovine incisors was obtained with the presence of the smear layer (Lalh et al. 1999).

Different intraoral conditions and dentin types affected the life-span of restorations when the smear layer was retained. Our experiments in sheep pointed to importance of adjustment of the smear layer as its preservation resulted in only 50% successfulness of Kavitan Plus GIC restorations after 9 months. Adjustment of dentin before placement of Vitremer restorations ensured 100% successfulness of treatment.

After 6 months of the experiment of the restorations with Kavitan Plus (smear layer retained) and Vitremer GIC (smear layer adjusted) in dogs exhibited 100% success. The adjustment of smear layer before placement of Kavitan Plus GIC was not inevitable. Retention of this layer (filling the orifice of dentinal tubules) may decrease the post-operative sensitivity important particularly in young animals having a very thin dentin layer lining the pulpal cavity.

Caries of classes A, D and F are most frequent in dogs. High degree of successfulness (82%) was reached used composites in the treatment of class A caries (Capík et al. 1999). The life-span of restorations used with class D caries was 6 months. Class F involved decay of dental tissue resembling osteoclastic resorption without presence of caries. Class D caries affect cemento-enamel junction and are associated with pulpal damage. The GIC used in our study in dogs proved successful in locations with higher humidity (cervical zone) owing to their hydrophilic character. Their ability to release fluoride ions prevents demineralisation of dentin and ensures its better regeneration. This was not observed with composites (Donly and Grandgenett 1998).

Veterinary practice in the country and abroad indicates that the category of pets includes besides carnivores, such as cats and dogs, also other animal species, e.g. herbivores, pigs and others. This wide spectrum of animals forces veterinary stomatologists to use restoration materials developed more likely for humans than for animals. Because of that we included in our experiment not only carnivores but also ruminants with such anatomy of dentition and processing of feed that can influence the overall use of GIC.

The results of our experiment proved that both conventional and resin-modified GIC can be used successfully in the treatment of dental cervical defects. No pathological intake and processing of feed was observed.

The use of GIC Vitremer and Kavitan Plus in veterinary stomatology is well-founded. They are advantageous particularly in locations in which the dry environment needed for application of amalgam and composites cannot be ensured. The most suitable location for application of GIC is the dental cervical zone. Neither cracks nor discoloration were observed in restorations. Present smear layer with Kavitan Plus was part of our experiment investigating the lifetime of restorations when the smear layer was retained in various types of dentitions and intraoral environments. It was based on the studies of Mitra and Conway (1994) and Puckett et al. (1995) who observed the lowest thermal expansion particularly with conventional GIC. Our results, particularly those in sheep, indicated the necessity of adjustment of the smear layer with a conditioner because in the 9 th month of the experiment two Kavitan Plus restorations showed signs of separation. In the 6th month of the experiment in dogs 100% successfulness of restorations with Kavitan Plus was observed. The size of microgaps measured at dog dentin was significant but resulted in no pathogenic states in the pulp or hard dental tissues.

Fluoride ions released from GIC support the treatment of dental hard tissues. These materials could be used as definitive restorations of class A and D cavities in dogs and dental cervical caries in sheep as well as underlying layers of composite and amalgam materials.

Hodnotenie okrajových netesností pri použití dvoch skloionomérnych cementov (SC) *in vivo* u psov a oviec v experimente

Cieľom experimentálneho štúdia bolo zhodnotiť parametre okrajových netesností dvoch skloionomérnych cementov: Kavitan Plus (Spofa Dental) a Vitremer (3M ESPE) *in vivo* na chrupe psov a oviec.

U psov a oviec sme *in vivo* použili klasický sklopolyalkenoátový chemicky tuhnúci cement s hydrofilnými vlastnosťami Kavitan Plus a živicovo-modifikovaný skloionomérny cement Vitremer, ktorý okrem svetelnej polymerizácie využíva autopolymerizačnú reakciu radikálov metakrylátových skupín bez prístupu svetla.

Parametre skloionomérov sme hodnotili v 6 skupinách zvierat po 2 ks v rôznych časových intervaloch (u psov po 1. 4. a 6. mesiaci a u oviec po 3. 6. a 9. mesiaci od začiatku experimentu). Výplne boli umiestnené na bukálnej ploche trvalého zubu. V daných intervaloch sme v celkovej injekčnej anestézii extrahovali u oviec (v jednej skupine) 4 rezáky a 4 črenovce (premoláre) a u psov (v jednej skupine) 6 rezákov a 4 premoláre. Počas celého experimentu sa v celkovej anestézii extrahovalo u oviec 24 zubov a u psov 30 zubov. Pri spracovaní vzoriek z psích zubov došlo k poškodeniu dvoch vzoriek. Histologické a štatistické údaje vychádzajú z počtu 28 zubov. U psov sme po 1. mesiaci zaznamenali jeden prípad uvoľnenia (vypadnutia) výplne Kavitanu Plus (úspešnosť výplne 80 %). U oviec sme po 9 mesiacoch experimentu zistili uvoľnenie dvoch výplní Kavitanu Plus (úspešnosť výplne 50 %). Výplne cementami Kavitan Plus a Vitremer boli počas experimentu bez prasklín a bez okrajových farebných zmien. Štatisticky významný rozdiel ($P = 0.04$) sa pozoroval pri dentíne u psov, kde pri výplniach s použitím skloionomérneho cementu Vitremer sme zaznamenali nižšie hodnoty okrajových netesností. Ostatné výsledky boli štatisticky nevýznamné (ANOVA test).

Podporná liečba tvrdých zubných tkanív je zabezpečená fluoridovými iónmi uvoľňovanými zo SC. Tieto materiály sa môžu použiť ako definitívne výplne krčkových kavít typu A až D u psov a krčkových kavít u oviec ako aj podložky pod kompozitné a amalgámové materiály.

Acknowledgement

The authors wish to express their thanks to the company AKVÁRIUM KOBOLKA Nové Zámky and Mrs. Cedidlová from DELIKA who kindly supplied granulated feed for the dogs during the experiment.

References

ABDALLA AI, ALHADAINY HA 1997: Clinical evaluation of hybrid ionomer restoratives in Class V abrasion lesions: Two-year results. Quintessence Int **28**: 255-258

ATTIN T, BUCHALLA W, KIELBASSA AM, HELLWIG E 1995: Curing shrinkage and volumetric changes of resin-modified glass ionomer restorative materials. Dent Mater **11**: 359-62

BUCHALLA W, ATTIN T, HELLWIG E 1996: Influence of dentin conditioning on bond strength of light-cured ionomer restorative materials and polyacid-modified composite resins. J Clin Dent **7**: 81-4

CAPÍK I 1996: Hypoplasia of enamel in dogs (In Slovak). Infovet **5**: 38-39

CAPÍK I 2005: Resistance of fractured canine teeth reconstructed by cast metal crowns with integrated post against pressure in vitro. Proceedings of the 14 th European Congress of Veterinary Dentistry, Ljubljana 2005, pp. 49-51

CAPÍK I, LEDECKÝ V, ŠEVČÍK A 1999: Non-traumatic diseases of dental hard tissues in dogs and possibilities of their therapy (In Slovak). Veterinářství **8**: 332-335

DONLY KJ, GRANDGENETT G 1998: Dentin demineralization inhibition at restoration margins of Vitremer, Dyract and Compoglass. Am J Dent **11**: 245-248

GLASPOOLE EA, ERICSON RL 1993: In vitro investigation of the caries inhibiting effect of fluoride releasing materials. J Dent Res **72**: abstr. 1448

HALE FA 1998: Dental caries in the dog. J Vet Dent **15**: 79- 83

HARVEY CE, EMILY PP 1993: Restorative Dentistry. Small Animal Dentistry. Mosby 1993, pp. 213-215

KHOUW-LIU VHW, ANSTICE HM, PEARSON GJ 1999: An in vitro investigation of a poly(vinyl phosphonic acid) based cement with four conventional glass - ionomer cements. Part 1: flexural strength and fluoride release. J Dent **27**: 351-357

LALH MS, TITLEY K, TORNECK CD, FRIEDMAN S 1999: The shear bond strength of glass ionomer cement sealers to bovine dentine conditioned with common endodontic irrigants. Int Endod J **32**: 430-435

MCLEAN JW, NICHOLSON JW, WILSON AD 1994: Proposed nomenclature for glass ionomer dental cement and related materials. Quintessence Int **25**: 587-589

MITRA SB, CONWAY WT 1994: Coeficient of thermal expansion of some methacrylate-modified glass-ionomer (abstract 944) . J Dent Res **73**: 219

NAJAR AL, SAQUY PC, VANSAN LP, SOUSA-NETO MD 2003: Adhesion of a glass- ionomer root canal sealer to human dentine. Aust Endod J **29**: 20-22

NEMEC A, PAVLICA Z, PETELIN M, ERŽEN D, CROSSLEY DA 2005: Fractured canine teeth in dogs in Slovenia: fracture types and treatment performed. Proceedings of the 14 th European Congress of Veterinary Dentistry. Ljubljana 2005, pp. 52-54

PUCKETT AD, FITCHIE JG, BENNETT B, HEMBREE JH 1995: Microleakage and thermal properties of hybrid ionomer restoratives. Quintessence Int **26**: 557-581

PARK SH, KIM KY 1997: The anticariogenic effect of fluoride in primer, bonding agent, and composite resin in the cavosurface enamel area. Oper Dent **22**: 115-20

RIGSBY DF, RETIEF DH, RUSSELL CM, DENYS FR 1990: Marginal leakage and marginal gap dimensions of three dentinal bonding systems. Am J Dent **3**: 289-294

TANUMIHARJA M, BURROW MF, TYAS MJ 2000: Microtensile bond strenght of glass ionomer (polyalkenoate) cement to dentine using four conditioners. J Dent **28**: 361-366

ACTA VET. BRNO 2006, 75: 411-417; doi:10.2754/avb200675030411

Cats in Czech Rural and Urban Households

E. BARANYIOVÁ[1], A. HOLUB, M. TYRLÍK[2], M. VOLFOVÁ[1]

[1]University of Veterinary and Pharmaceutical Sciences, Brno, [2] Masaryk University, Brno, Czech Republic

Received June 15, 2005
Accepted May 11, 2006

Abstract

Baranyiová E., A. Holub, M. Tyrlík, M. Volfová: Cats in Czech Rural and Urban Households. Acta Vet. Brno 75, 2006: 411-417.

The aim of this study was to elucidate the effects of rural and urban environments on the co-existence of humans and their cats. From the obtained questionnaire data we selected the rural cats (R, n = 54) and compared them with urban cats (U, n = 144). The R group cats lived predominantly in family houses, U cats in urban apartments.

The pressures of physical and social factors in the small niches of urban apartments (dwellings in Czech urban high-density living settings, though comfortable, are smaller than in numerous European countries; they prevailed in our U group) resulted in statistically significant differences in only 31 (51.7%) out of 60 traits under study. Among them, 15 (68.2%) out of 22 concerned the conduct of household members, and 16 (42.1%) out of 38 concerned the behaviour of their cats. Thus the conduct of people in U households showed relatively higher proportion of changes than the behaviour of their cats. U onwers more frequently purchased their cats (R = 24.1%, U = 48.6%, chi-square = 10.648, df = 4, $p < 0.05$), they kept the cat pedigrees (R = 37.0%, U = 75.4%, chi-square = 24.661, df = 1, $p < 0.001$), paid more attention to their cats ((R = 93.0%, U = 100.0%, chi-square = 8.950, df = 1, $p < 0.005$), talked to them daily (R = 87.0%, U = 98.6%, chi-square = 12.024, df = 1, $p < 0.001$), allowed them to use furniture (R = 77.8%, U = 100.0%, chi-square = 33.839, df = 1, $p < 0.001$), sleep in beds of family members (R = 61.1%, U = 95.1%, chi-square = 37.149, df = 1, $p < 0.001$), and celebrated their birthdays (R = 25.9%, U = 100.0%, chi-square = 7.014, df = 2, $p < 0.05$). Their cats were more destructive than R cats, hunted less and were less aggressive when stroked. However, they showed a slightly larger scope of aggressive behaviours and were more frequently described as nervous and restless.

The nature of the significant differences found in this study indicates that the co-existence of cats with people in the urbanized world is becoming more intimate. More interactions occur between the two species. The relationships between people and their cats deserve further study not only in order to gain more insight but also for a practical application of this knowledge.

Questionnaire, behaviour, human-cat interactions, housing,

In the information age of North America and Western Europe increasingly more cats are kept (Anon 1990; Bergler 1991; Nott 1996; APPMA 2006; Rochlitz 2000a; Voigt 2000; US Pet Ownership and Demographic Sourcebook 2002). This is also true of the Czech Republic (Turner and Bateson 2000; Mahelka 2004; Baranyiová et al. 2004b). Whereas in rural areas cats are mostly kept in family houses, in towns and cities they often live in relatively small apartments. This affects their interactions with household members. The human-cat bond may be stronger or weaker, depending on the roles that cats play in the household activities. For example, in rural areas they are expected to protect the household from rodents whereas in towns and cities they are perceived as companions and share permanently the apartment niches with their owners (Baranyiová et al. 2004b).

More detailed knowledge of the consequences of human and cat co-existence under these two different conditions is still rather fragmentary and modest (Bergler 1988, 1991; Turner 1991, 1995, 2000; Beaver 1992; Houpt 1998; Turner and Rieger 2001; Baranyiová et al. 2004b; Rochlitz 2005). We therefore decided to further explore and elucidate this bond.

Address for correspondence:
Doc. MVDr. E va Baranyiová, CSc.
University of Veterinary and Pharmaceutical Sciences
Palackého 1-3, 612 42 Brno
Czech Republic

Phone: +420 541 562 791
E-mail: actavet@vfu.cz
http://www.vfu.cz/acta-vet/actavet.htm

Materials and Methods

We analysed data obtained by a questionnaire based on Askew (1997) and Bergler (1991) published in the monthly magazine "Naše kočky" (Our Cats). Its readers provided about 90% of the data. The rest was obtained from clients of veterinary practices who were contacted personally (Baranyiová et al. 2004b). We thus evaluated the behaviour of 198 cats whose owners provided information about their housing type. Group 1 comprised 54 cats living in rural areas (R), group 2 was formed of 144 cats living in urban settings (U).

The questionnaire had 60 questions that were in part binary, in part multiple. They can be summarized into several groups:

a) data on age, sex and breed of the cat, age at acquisition and health status,

b) data on the structure of the household (number of adults and children, species and numbers of other animals), housing style,

c) the daily regime of cats (including their nutrition),

d) other behaviours of cats, including the negatively perceived ones such as aggression,

e) evaluation of the overall position of cats in the households.

We were mainly interested in how frequently the behaviours were observed and whether there were significant differences between rural and urban cats. Per cent proportions were calculated from the actual numbers of owner answers and not from the frequencies of groups. Analysis of contingency tables was carried out using χ^2 test (statistical software SPSS v. 8).

Results

In both groups, R and U, we compared 60 traits, behaviours of cats and conduct of their owners.

The compared groups did not differ significantly in numbers of household members except for the fact that in group R no one-member household was reported (R = 0.0%, U = 25.2%, χ^2 = 10.114, df = 2, $p < 0.01$), whereas in group U these households comprised one fifth. Moreover, they consisted exclusively of women. In R areas, also other animals were kept more frequently with cats (R = 15.1%, U = 4.3%, n.s.), and dogs (R = 34.0%, U = 17.3%, χ^2 = 16.230, df = 3, $p < 0.001$). U cats thus lived in a less complex social environment.

We evaluated 22 conduct traits of household members. Their responses indicate that sex distribution of cats in R and U groups did not differ significantly: the proportion of tomcats (R = 26.4%, U = 17.8%), female cats (R = 41.5%. U = 45.9%) or neutered animals (R = 32.2%, U = 36.2%). Similarly, kittens did not differ in age at which they were adopted into R or U households. Most of them were adopted before 10 weeks of age (R = 59.3%, U = 46.3%), fewer before they reached six months (R = 27.8%, U = 44.1%) and only the rest of them at an older age (R = 13.0%, U = 9.6%). We found a significant association between the type of housing and the way in which the cat was acquired. In rural areas only one fourth of cats were purchased whereas in urban areas is was nearly a half of them (R = 24.1%, U = 48.6%, χ^2 = 10.648, df = 4, $p < 0.05$). In the R environment significantly fewer people cared for the origin of their cats. Only about one third of these animals were pedigree cats whereas in urban areas three quarters of cats were purebreds with a pedigree (R = 37.0%, U = 75.4%, χ^2 = 24.661, df = 1, $p <$ 0.001). A significant association was found between the age and morbidity of R and U cats. Up to the age of six months, rural cats were sick more frequently (R = 28.6%, U = 3.8%); whereas urban cats suffering from diseases were three and more years of age (R = 19.0%, U = 46.2%, χ^2 = 7.289, df = 2, $p < 0.05$).

Cat owners reported that more than four fifths of cats in both groups had toys and scratching posts (R = 83.3%, U = 89.1%). There were no differences in frequency and length of time that the household members spent playing with their cats (R = 83.3%, U = 94.4%).

When analyzing 38 traits of cat behaviour we found no differences except for one case of feeding behaviour; R cats were hunting more frequently (R = 16.7%, U = 2.1%, χ^2 = 113. 978, df = 8, $p < 0.001$). Both groups equally soiled the living quarters, defecated (R = 7.4%, U = 5.8%), urinated (3.7%, U = 6.3%) and urine-marked (R = 1.9%, U = 1.4%). In urban cats, though rarely, pathological behaviours were reported such as excessive nibbling/biting and licking of haircoat (R = 0.0%, U = 0.7%) and insufficient grooming

(R = 0.0%, U = 0.7%), whereas in R cats these behaviours were not found at all. Urban owners complained significantly more often than the rural ones about their cats scratching furniture and carpets (R= 56%, U = 25.2%, $\chi^2 = 9.503$, df = 1, $p < 0001$).

Analysis of feline aggression indicated that cats of the two groups did not differ in their interspecies aggression (R = 18.5%, U = 11.9%, n.s.). Rural cats were more frequently aggresssive when petted (R = 5.6%, U = 0.7%, $\chi^2 = 4.647$, df = 1, $p < 0.05$), but they showed no aggression at all when punished or reached toward, and were not aggressive to unknown persons or household children (all R = 0.0%, U = 0.7%).

The respondents made conclusions about the character and nature of their U and R cats. More than nine tenths of cats were described as able to perceive the moods of household members (R = 90.7%, U = 96.5%), four fifths of them were considered cuddly (R = 81.5%, U = 81.8%) and about two thirds of them were described as playful (R = 63.0%, U = 70.6%). About one half of the cats were considered independent (R = 51.9%, U = 42.7%). Other traits were found rarely. For example, excessive locomotor activity (R = 16.7%, U = 11.9%) or hypoactivity (R = 9.3%, U = 7.7%) was reported in both groups only in about one tenth of the cats. In the R group, not a single cat was described as nervous or restless.

On the other hand, in data on the position of cats in households significant differences were found more often. In U households they were given more attention (R = 93.0%, U = 100.0%, $\chi^2 = 8.950$, df = 1, $p < 0.005$), household members spoke to their cats (R = 87.0%, U = 98.6%, $\chi^2 = 12.024$, df = 1, $p < 0.001$), tolerated their use of furniture (R = 77.8%, U = 100.0%, $\chi^2 = 33.839$, df = 1, $p < 0.001$), sleeping in beds of family members (R = 61.1%, U = 95.1%, $\chi^2 = 37.149$, df = 1, $p < 0.001$), and they celebrated the cats' birthdays (R = 25.9%, U = 100.0% $\chi^2 = 7.014$, df = 2, $p < 0.05$).

When the expected and observed frequencies in the individual fields of the contingency tables were compared, we found statistically significant differences in about one half of the traits under study, i.e. in 31 out of 60 (51.7%). The physical and social pressures in the small niches of urban apartments manifested themselves significantly. They were more frequently found in the conduct and actions of household members - in 15 (68.2%) out of 22 cases, i.e. in two thirds of traits. In the evaluation of cat behaviour this was true in 16 (42.1%) out of 38 cases, i.e. in two fifths of traits. In the activities of urban household members we thus found relatively more changes (by 1/4) than in the behaviour of their cats.

Discussion

Urbanization of the Czech society has several specific features. For example, high-density living settings making up a large proportion of urban dwellings, may be technically well equipped and comfortable for people, yet they are smaller than those in other European countries (Baranyiová et al. 2005). Moreover, in these small urban niches indoor cats are kept for long periods, often for a whole lifetime. These physical factors impose an inevitable strong pressure on them.

Cats are exposed to other, social influences, too: for example, urban households in our study were also one-person households and consisted exclusively of women. Hence women seem to select cats for their companions more frequently. It is generally assumed that the cat-human relationship is more intense, when the human partner is female (Turner et al. 2003; Adamelli et al. 2005). Cats themselves contribute to the closeness of this bond. This is perhaps because usually women take care of the cats, and feed them (Bradshaw 1992). Nevertheless, this problem has not been sufficiently elucidated and the opinion of Adamelli et al. (2005) can be supported that it needs further investigation in order to clarify whether women are really more involved in interactions with cats than men. Apart from this, in our U households there were fewer other animals kept. Thus cats living in these households had fewer social interactions.

These unusual and unprecedented physical and social pressures in U households significantly affected only a few (two fifths) of the feline behaviour traits under study (Baranyiová et al. 2003ab, 2004ab). Cats show less exploratory activity, they leave the apartments less frequently, roam less and hunt rarely. They tolerate better unsolicited physical contact and stroking. However, they are more often described as nervous and restless. This fact may be the result of lack of physical activity, since rural cats may have territories as large as 200 (females) and 600 hectares (males), as reported by Turner and Bateson (1988, cited in Houpt 1998). In our U cats, grooming tended to be either excessive or neglected. They also showed several types of aggression not encountered in R cats.

They significantly more often scratched furniture and carpets, soiled the apartment though not as frequently as reported in Great Britain where house soiling (indoor marking) is considered as the most frequent problem reported by cat owners, making up 25% of the problems seen (Anon 2002; Hoole 2004).

Interestingly, the cat owners' conduct was changed more than the behaviour of pet cats in U households. We found a similar trend in households with dogs (Baranyiová et al. 2005). Human-cat interactions in U and R conditions differed significantly in two thirds of the indicators under study. When selecting their cats, urban people act differently than rural owners: they more often purchase the cats, keep their pedigree, pay more attention to them, talk to them daily, allow them to use furniture and sleep in beds. They celebrate the cats' birthdays and, generally, show a higher degree of attachment to them.

In recent decades, these problems, namely the effects of various physical and social variables on the behaviour of cats are given more attention from multiple approaches. However, the data are often based only on surveys of their housing in laboratories, quarantine stations and shelters (McCune 1995; Rochlitz et al. 1998; Ottaway and Hawkins 2003; Rochlitz 2005). They are sometimes connected with the "The Five Freedoms" of the Brambell report (1965), with the attempts to enrich their environment and improve welfare (Turner 1995; Scott et al. 2000; Young 2003) and the aim of preventing behavioural problems (Schroll 2002). Fewer are directly aimed at interactions of humans and cats in households (Mertens and Turner 1988; Mertens 1991; Rochlitz 2005) and on problems of outdoor and indoor housing. Most authors agree that cats adapt well to life in urbanized environment, especially when they are raised indoors from an early age (Rochlitz 2005). Our observations also demonstrate great adaptability of cats living in urban environments as evidenced by the frequency of some of their behavioural traits being significantly different from those of the rural cats. However, cats that began to live in small urban apartments later in their lives, at an older age, may have some behavioural problems (Hubrecht and Turner 1998).

Some authors draw attention to differences between outdoor and indoor cats in conditions and risks of disease (Buffington 2002) . This fact may be connected with our findings on significant differences in age distribution of morbidity between R and U cats; R cats tended to be more frequently sick before 6 months of age whereas more U cats when sick, were older than 3 years.

It is necessary to note that the respondents to our questionnaire represent that portion of Czech population that has a positive attitude to cats. They are interested in their cats, observe them carefully, and are able to formulate their views about the behaviour of the cats. Some of them read regularly cat magazines, participate in internet chats about cats and seek professional help when necessary. Therefore they cannot be considered as fully representative of the human population of the country. Also in the Czech Republic the views about keeping cats are somewhat polarised and controversial (Baranyiová et al. 2003ab, 2004ab). It may be assumed that some portion of the population does not like cats.

Domestication of cats may be interpreted as a process that began in the Middle East

thousands of years ago by mutual approach of the two species when crop protection from rodents became a necessity. Cats were able to retain their relative independence from people. Their domestication, however, may be seen in a different way. They began to lay and rest near stoves and purr in their owners' laps much later, and only recently they have been given the chance to enjoy the comfort of our urbanized homes. Therefore they are sometimes considered as domesticated as late as during the last 150 years (Bökönyi 1989; Serpell 2000). Their domestication continues presently, in different ways in differring environments. Their behaviour continues to show obvious signs of independence and self-reliance, and their locomotor behaviours are highly variable. They have many specific individual features and differ from other types of human-animal interaction (Bradshaw 1992). It makes the interpretation of their behaviour more difficult. Therefore exact observations of feline locomotion are difficult to interpret and generalize.

Moreover, cats continue to be encompassed with controversial tales, myths and prejudices, by their opponents and lovers alike. They are considered to be independent, unpredictable, false, but also charming, cuddly, reserved and shy. It is possible that such anthropomorphism makes itself felt also in the views of our respondents. More thorough knowledge and objective understanding and interpretation of feline behaviour and their relationships to humans need further efforts (Baranyiová et al. 2004b).

Kočky v českých venkovských a městských domácnostech

Ve snaze zjistit, jak se vliv venkovského a městského prostředí uplatňuje v soužití lidí a koček v českých domácnostech, oddělili jsme ze souboru koček, kterým se opakovaně zabýváme, kočky městské (U, n = 144) a srovnávali je s venkovskými (R, n = 54). Skupina R žila převážně v rodinných domcích se zahradou, skupina U v městských bytech. Data jsme získávali pomocí dotazníků.

Fyzikální a sociální tlak urbanizovaného prostředí, malých nik městských bytů (české sídlištní byty, které v městské skupině převažují, jsou sice komfortní, ale mají menší plochu než v četných zemích Evropy), vyvolávají statisticky významné rozdíly jen u 31 (51,7%) ze 60 sledovaných znaků. Z toho jich 15 (68,2%) ze 22 připadá na jednání členů domácností a 16 (42,1%) ze 38 na chování koček. Jednání lidí v U domácnostech tudíž vykazuje relativně větší počet změn než chování jejich koček. Městští chovatelé si kočky průkazně častěji kupují polovina (R = 24,1%, U = 48,6%, $\chi^2 = 10,648$, df = 4, $p < 0,05$), mají doklady o jejich původu (R = 37,0%, U = 75,4%, $\chi^2 = 24,661$, df = 1, $p < 0, 001$), nechovají doma další živé tvory , více si svých koček všímají (R = 93,0%, U = 100,0%, $\chi^2 = 8,950$, df = 1, $p < 0,005$), častěji na ně mluví (R = 87,0%, U = 98,6%, $\chi^2 = 12,024$, df = 1, $p < 0,001$), slaví jejich narozeniny (R = 25,9%, U = 100,0% $\chi^2 = 7, 014$, df = 2, p < 0,05), dovolují jim využívat bytové vybavení zařízení (R = 77,8%, U = 100,0%, $\chi^2 = 33,839$, df = 1, $p < 0,001$) a spát v postelích postelích (R = 61,1%, U = 95,1%, $\chi^2 = 37,149$, df = 1, $p < 0,001$). Jejich kočky více destruují zařízení domácností, škrábou po nábytku a kobercích, méně loví a bývají méně agresivní při hlazení. Vykazují však širší, byť nevelké, spektrum agrese a bývají označovány za nervózní a neklidné.

Povaha průkazných odchylek vesměs dokládá, že soužití koček s lidmi je v urbanizovaném světě těsnější. Interakcí lidí a koček přibývá. Kočky své malé zatěžující niky téměř neopouštějí a mimo ně jeví menší explorativní aktivitu. Zkoumání vztahů lidí a koček v urbanizovaném světě je žádoucí v zájmu jejich hlubšího poznání i praktického využití zintenzivňovat.

Acknowledgement

The stuy was partly supported by the MŠMT projects FRVŠ 1471/2004 and 1823/2005.

References

ADAMELLI S, MARINELLI L, NORMANDO S, BONO G 2005: Owner and cat features influence the quality of life of the cat. Appl Anim Behav Sci **94**: 89-98

Anonymous 1990: Cats across the world. Anthrozoös **3**: 196

Anonymous 2002: Behavioural problems in cats and dogs. Vet Rec **151**: 252

APPMA: 2005-2006 National Pet Owners Survey. American Pet Product Manufacturers Association Inc. Greenwich 2006. LXIII and 376 p.

ASKEW HR: Behandlung von Verhaltensproblemen bei Hund und Katze. Paul Parey, Berlin 1997. VII a 372 p.

BARANYIOVÁ E, HOLUB A, JANÁČKOVÁ B, ERNSTOVÁ M 2001: Výpovědi chovatelů o psech v České republice. Veterinářství **51**: 167-171

BARANYIOVÁ A, HOLUB A, VOLFOVÁ M, TYRLÍK M, LIČKOVÁ A, SAMKOVÁ A 2003a: Psi a kočky v českých domácnostech - srovnání. (Dogs and cats in Czech households - a comparison) In: 30. etologická konference. Program a abstrakta. Jičín. p. 42

BARANYIOVÁ A, HOLUB A, VOLFOVÁ M, TYRLÍK M, LIČKOVÁ A, SAMKOVÁ T 2003b: Psi a kočky v českých domácnostech. (Dogs and cats in Czech households). In: Ochrana zvířat a welfare 2003, část A. Brno. p. 8-12

BARANYIOVÁ A, HOLUB A, VOLFOVÁ M, TYRLÍK M, LIČKOVÁ A, SAMKOVÁ T 2004a: Kočky v našich domácnostech. (Cats in our households). In: 31. etologická konference. Program a abstrakta. Poľana. p. 43

BARANYIOVÁ E, HOLUB A, VOLFOVÁ M, TYRLÍK M, LIČKOVÁ A, SAMKOVÁ A 2004b: Výpovědi respondentů o kočkách v České republice. (Responses of owners about cats in the Czech Republic). Veterinářství **54**: 490-496

BARANYIOVÁ E, HOLUB A, TYRLÍK M, JANÁČKOVÁ B, ERNSTOVÁ M 2005: The influence of urbanization on the behaviour of dogs in the Czech Republic. Acta vet Brno **74**: 401-409

BEAVER BV 1992: Feline Behavior: A Guide for Veterinarians. W.B. Saunders, Philadelphia, 276 p.

BEAVER BV 2002: Behavioural problems in cats and dogs. Vet Rec **151**: 252

BERGLER R 1988: Die Psychologie der Beziehungen von Heimtieren, Heimhalter und Tierärzten. J Vet Med B **35**: 443-461

BERGLER R 1991: Man and Cat. Blackwell Scientific Publications. London. 120 p.

BÖKÖNYI S 1989: Definition of animal domestication. In: The Walking Larder: patterns of domestication, pastoralism, and predation. London p. 22-27

BRADSHAW JWS 1992: The cat-human relationship. In: The Behaviour of the Domestic Cat. Wallington, UK, CAB International, p. 163-176

BRAMBELL FWR 1965. Report of the Technical Committee to Enquire into the Welfare of Livestock Kept Under Intensive Husbandry Systems. London

BUFFINGTON CAT 2002: External and internal influences on disease risk in cats. J AmerVet Med Assoc 22**0**: 994-1002

HOOLE J 2004: Annual Review of Cases 2003., accessed May 21, 2004

HOUPT KA 1998: Domestic Animal Behavior for Veterinarians and Animal Scientists. Third edition. Iowa State University, Ames. 495 p.

HUBRECHT RC, TURNER DC 1998: Companion animal welfare in private and institutional settings. In: Companion Animals and Human Health. Thousand Oaks. p. 267-289

MAHELKA B 2004: Personal communication

MCCUNE S 1995: Enriching the environment of the laboratory cat - a review. In: Proceedings of the Second International Conference on Environmental Enrichment. Copenhagen. pp. 103-117

MERTENS C 1991: Human-cat interactions in the human setting. Anthrozoös **4**: 214-231

MERTENS C, TURNER DC 1988: Experimental analysis of human-cat interaction during first encounters. Anthrozoös 2: 83-97

NOTT HMR 1996: Eurocats. J Feline Advis Bureau **34**: 46-47

OTTWAY DS, HAWKINS DM 2003: Cat housing rescue shelters: a welfare comparison between communal and discrete-unit housing. Anim Welfare **12**: 173-189

ROCHLITZ I 2000a: Feline welfare issues. In: TURNER DC, BATESON P (Eds): The Domestic Cat. The biology of its behaviour. Second edition. Cambridge University Press, Cambridge, pp. 207-226

ROCHLITZ I 2000: Recommendations for the housing and care of domestic cats in laboratories. Lab Animals **34**: 1-9

ROCHLITZ I 2005: A review of the housing requirements of domestic cats (*Felis silvestris catus*) kept in the home. Appl Anim Behav Sci **93**: 97-109

ROCHLITZ I, PODBERSCEK AL, BROOM DM 1998: The welfare of cats in a quarantine cattery. Vet Rec **143**: 35-39

SCHROLL S: Environmental enrichment for indoor cats as prevention and therapy-practice advise for quality of life. In: Proceedings of the Companion Animal Behaviour Therapy Study Group. Birmingham 2002. p. 43-45

SCOTT PW, STEVENSON MF, COOPER JE, COOPER ME: Secretary of State#s Standards of Modern Zoo Practice. Norwich 2000

SERPELL JA: Domestication and history of the cat. In: TURNER DC, BATESON P (Eds): The Domestic Cat. The biology of its behaviour. Second edition. Cambridge University Press, Cambridge 2000. p. 179-192
TURNER DC, BATESON P 1988: The Domestic Cat. The Biology of Its Behaviour. Cambridge University Press
TURNER DC 1991: The ethology of the human-cat relationship. Schw Arch Tierheilk **133**: 63-70
TURNER DC 1995: Ethologie und Wohlbefinden bei Heimtieren. Schw Arch Tierheilk **137**: 45-49
TURNER DC 2000: The human-cat relationship. In: TURNER DC, BATESON P (Eds): The Domestic Cat. The biology of its behaviour. Second edition. Cambridge University Press, Cambridge, pp. 193-206
TURNER DC, BATESON P 2000: Why the cat? In: TURNER DC, BATESON P (Eds): The Domestic Cat. The biology of its behaviour. Second edition. Cambridge University Press, Cambridge, pp. 3-6
TURNER DC, RIEGER G 2001: Singly living people and their cats: A study of human mood and subsequent behavior. Anthrozoös **14**: 38-46
TURNER DC, RIEGER G, GYGAX L 2003: Spouses and cats and their effects on human mood: Anthrozoös 1**6**: 213-228
U. S. Pet Ownership & Demographics Sourcebook. Schaumburg 2002. 46 p.
VOIGT A: 2000 AVMA Directory and Resource Manual. AVMA, Schaumburg 2000. 1022 p.
YOUNG RJ: Environmental Enrichment for Captive Animals. Oxford 2003

ACTA VET. BRNO 2006, 75: 419-425; doi:10.2754/avb200675030419

The Effect of Sterilization on Size and Shape of Fat Globules in Model Processed Cheese Samples

B. TREMLOVÁ[1], P. ŠTARHA[2], F. BUŇKA[3], Z. GISTINGROVÁ[1], J. HRABĚ[3]

University of Veterinary and Pharmaceutical Sciences Brno[1)]
Brno University of Technology[2)]
Tomáš Baťa University in Zlín[3)]

Received November 3, 2005
Accepted May 11, 2006

Abstract

Tremlová B., P. Štarha, F. Buňka, Z. Gistingrová, J. Hrabě: The Effect of Sterilization on Size and Shape of Fat Globules in Model Processed Cheese Samples. Acta vet. Brno 2006, 75: 419-425.

Model cheese samples from 4 independent productions were heat sterilized (117 °C, 20 minutes) after the melting process and packing with an aim to prolong their durability. The objective of the study was to assess changes in the size and shape of fat globules due to heat sterilization by using image analysis methods. The study included a selection of suitable methods of preparation mounts, taking microphotographs and making overlays for automatic processing of photographs by image analyser, ascertaining parameters to determine the size and shape of fat globules and statistical analysis of results obtained.

The results of the experiment suggest that changes in shape of fat globules due to heat sterilization are not unequivocal. We found that the size of fat globules was significantly increased ($p < 0.01$) due to heat sterilization (117 °C, 20 min), and the shares of small fat globules (up to 500 μm^2, or 100 μm^2) in the samples of heat sterilized processed cheese were decreased. The results imply that the image analysis method is very useful when assessing the effect of technological process on the quality of processed cheese quality.

Processed cheese, heat sterilization, fat, image analysis, histochemical analysis

Image analysis methods have become a useful tool for the evaluation of various defects and quantitative variables of food materials. The measurement of different parameters using image analysis is associated with important sensoric and technological properties of food materials when it comes to food samples. The study by Holcomb et al. (1992) showed that on the basis of rheological and structural examinations of dairy products, functional properties of products can be estimated by evaluating their microscopic structure. Also in the study of dairy products, image analysis methods are used, often associated with the microscopic and sensoric evaluation.

Caccamo et al. (2004) compared the formation of gases in different kinds of cheese, focusing on the measurement of gas holes size in the section area. These methods provide a considerable potential for the evaluation of processed cheese quality, mainly in association with sensoric analysis. Thus, it is possible to estimate structural changes due to different heat treatments that affect the compact nature of protein matrix or fat particle distribution, which are the factors unequivocally influencing the texture of processed cheeses (Caric and Kaláb 1997; Guinee 2003).

Processed cheeses are manufactured by heating a mixture of various kinds of natural cheeses at different stages of maturity, with emulsifying agents under partial vacuum and constant stirring, until a homogenous blend of required properties is achieved. Other raw materials, both dairy and non-dairy ones, can be added to the blend of natural cheeses.

Address for correspondence:
Doc. MVDr. Bohuslava Tremlová, Ph.D.
Department of Vegetable Foodstuffs and Plant Production
University of Veterinary and Pharmaceutical Sciences Brno
Palackého 1-3
612 42 Brno, Czech Republic

Phone: +420 541 562 700
E-mail : tremlovab@vfu.cz
http://www.vfu.cz/acta-vet/actavet.htm

Phosphates, polyphosphates, and, to a smaller extent, citrates are used as emulsifying agents (Caric and Kaláb 1997).

When soldiers cannot be provided with warm food (separation from the unit, combat, etc.), they can receive so-called combat rations. There are special requirements for minimum shelf life of food components of combat rations. The minimum shelf life of these was determined as 24 months at the ambient temperature (Standardization Agreement No. 2937, 2001). The combat rations can also be used by members of the integrated rescue system when in operation. Combat rations of NATO armies (e.g. U.S.A., Germany and France) contain processed cheese.

Processed cheese is a typical non-acid food. It is usually melted under 100 °C (Bylund 1995). The temperature can only kill vegetative forms of microorganisms but not bacterial spores that may cause degradation of the product (Mafart et al. 2001). One of the ways to achieve a shelf life of 24 months in processed cheeses is heat sterilization.

Yoon and McCarthy (2003) studied properties of processed cheese samples by using a combined method of magnetic resonance and image analysis with different melting conditions. In the study by Wang and Sun (2002), a computerized system of image analysis was used for the evaluation of cheese properties, relevant for the melting process. For the comparison, two kinds of cheese were chosen (Cheddar and Mozzarella) and various combinations of time and temperature were used. Wang and Sun (2003) used the image analysis for Cheddar and Mozzarella cheeses once again. This time the purpose was to determine colour changes in these two cheeses during the heating. When the course of browning process was evaluated, it was found that correlations between time and temperature defined as browning factor are different. The method used proved to be an efficient and objective tool for monitoring colour changes in cheese.

The properties of processed cheese are influenced by many factors such as the composition, kind and degree of maturity of the cheese used, as well as of the added components, the nature and amount of emulsifiers, pH and manufacturing process parameters (Marchesseau et al. 1997). Some researchers (Raval and Mistry 1999) stated that milk fat is a very important component of processed cheeses since it influences their properties, for instance spreadability.

In the study performed by Klostermeyer and Buchheim (1988), samples withdrawn at different stages of the melting process were investigated. Using electron microscopy, they studied the microstructure and compared the structure of protein matrix and its changes during the melting processes. They used two samples of processed cheese, made under the same conditions, the only difference being the rotation rate of the stirring device (lower and higher (10 ×) rotation rate). Differences in the size of fat globules were obvious, and much smaller at a higher rotation rate. There were some differences within the protein matrix, too, and the samples processed under the higher rotation rate of the melting boiler showed locally markedly larger protein particles.

Sutheerawattananonda et al. (1997) studied the effect of heat treatment duration on fat particle distribution and the degree of emulsification of processed cheese samples, using various kinds of emulsifying agent. They measured the size and shape of fat particles in model samples of processed cheese, using fluorescence microscopy and image analysis. Different heat treatments and the presence of emulsifying agents significantly influenced the shape and size of fat globules. Results showed that there were not any truly round or circular globules in the cutaway view. For the examination by the fluorescence microscope, the samples were stained with Nile blue. For the light microscopy, methods using the Sudan group stains, Oil red and osmium tetroxide are most commonly used. The rate and intensity of staining depend on the stain concentration (Flint 1994).

Awad et al. (2002) compared the texture and microstructure of cheese samples processed with different emulsifying agents. Different manufacturing process conditions influenced

the number and size (diameter) of fat globules. The size (diameter) ranged from 16.9 ± 0.5 to 87.5 ± 7.9 μm in different samples. Marchesseau et al. (1997) evaluated the effect of pH value on the microstructure of processed cheeses, mainly in terms of changes in the protein matrix. They found fat globules of 2 - 3 μm in diameter.

Bowland and Foegeding (2001) monitored the effect of different processing conditions on rheological properties and properties of the lipid phase in model samples. Using microscopic examination and image analysis they found that percentages of the cross-sectional area took up by fat in model samples ranged from 31.0 ± 4.9 to 41.5 ± 6.5%. The size (diameter) of fat globules ranged between 3.53 ± 0.49 and 5.45 ± 0.84 μm.

Many reported results suggest that image analysis is suitable for quantitative evaluation of images obtained by microscopic examination (e.g. Bijker et al. 1983; Hildebrandt and Hirst 1985; Rao et al. 1989; Heertje and Leunis 1997; Palka and Daun 1999). Depending on the aim of examination, variables to be measured are chosen. The aim of examination is often to ascertain the size, shape or colour of objects (Zikmundová 1996). The basis for processing and evaluation of a microscope image is a sufficient contrast enabling the analyser to identify objects correctly. This depends both on the staining method chosen and the quality of mount (Tremlová and Štarha 2002).

The objective of the present study was to apply the method of image analysis of microscope mount to assess the effect of heat sterilization treatment (117 °C, 20 min) on the size and shape of fat globules in model processed cheeses.

Materials and Methods

Four groups of processed cheese were analysed. The values declared were dry matter (38% w/w) and fat on dry matter basis (45% w/w). Each group of processed cheese was manufactured separately from other groups, but using the same technological procedure. The processed cheeses were made from a mixture of natural cheeses, butter, cottage cheese, water and emulsifying agents. The melt was filled in laminated aluminium containers. After closing the containers, each group of samples was divided into two parts. The first half of samples from all the groups was cooled down to 10 °C within 2 hours (hereinafter referred to as "non-sterilized processed cheeses" - N); the second half was subjected to sterilization treatment. Heat sterilization was performed by the sterilizer LUBECA at 117 °C for 20 minutes; then the samples in the sterilizer were cooled down to 25 °C (hereinafter referred to as "sterilized processed cheeses" - S). The samples were stored in the refrigerator at 8 ± 2 ºC, until the analyses were carried out. Each time, 3 packages of non-sterilized and 3 packages of sterilized processed cheese were analysed in each group.

For the image analysis, microscope mounts were made and stained with Oil red (Plate V, Fig. 1). The mounts were examined by the microscope Jenaval 250-CF, using 32-fold magnification. Microphotographs were taken by the digital camera Olympus C 4000. The documentation for each sample included about 40 pictures, and for image analysis, 16 microphotographs were chosen from each picture and individually processed by the image analysis software ACC (Image Structure and Object Analyse, v. 6.0). The processing of pictures was based on the scheme presented in the study by Tremlová and Štarha (2002) and included the following steps:

1 Insertion of a measure for the magnification used
2 Creation of an overlay for fat globules, based on the colour and brightness of standard samples selected
3 Determination of variables (area of objects, SF = shape factor, sizes of half axes A and B)
4 Analysis (automatic, using an overlay, manual corrections).

The evaluation of shape was based on the values of the size of half axes of the Legendre ellipse which approximates the object measured, and the shape coefficient calculated. The changes in shape were evaluated by comparing mean values of shape coefficient t_{ab} for different samples which was determined according to the equation (1). When the shape of an object resembles a circle, the value of shape coefficient approaches k + ∞, when the shape of an object resembles a line, the shape coefficient value approaches k - ∞. A basic property of the Legendre ellipse is that its area is the same as that of the object analysed.

$$t_{ab} = -\ln\left(\frac{a^6 + b^6}{a^3 b^3} - 2\right) \quad (1)$$

a: length of the major half axis
b: length of the secondary half axis
ln: hyperbolic logarithm.

The size of fat globules was determined as a value obtained by measuring their area.

Student's *t*-test at a significance level of $\alpha = 0.01$ was used to evaluate whether there were any statistically significant differences in the shape and size of fat globules between the samples of non-sterilized processed cheeses and sterilized processed cheese. In order to simplify the situation, we anticipated normal distribution of shape coefficient $t_{a,b}$ values.

For comparison, the non-parametric Wilcoxon test (Agresti 1987) was used to assesses whether differences in the shape and size of fat globules between the samples of non-sterilized processed cheese and sterilized processed cheese were significant or not. The change in fat globule size distribution was also evaluated by calculating percentages of small objects before and after the heat treatment. The Microsoft EXCEL software was used to perform the calculations.

Results

Table 1 gives basic results found by image analysis of samples. For each sample, the same number of pictures (16) was analysed and total image area was the same for all the samples, too.

Table 1. Summarized results of the examinations

Group	I		II		III		IV	
	N	S	N	S	N	S	N	S
Parameter								
No. of pictures	16							
Globule area								
∑ mm²	0. 73	1.16	0.72	0.55	1.75	2.21	4.64	0.59
Smallest globule µm²	10.5	36.7	10.5	10.5	10.5	10.5	12.2	10.5
Largest globule µm²	8070.9	27936.4	17446.8	3104.3	8657.0	42677.2	2320.8	13504.8
Globule count	1671	2179	1875	1515	3564	3353	2288	2193

N: non-sterilized, S: sterilized

Table 2 presents general results of shape evaluation of different groups of processed cheeses. The statistical analysis (Student's *t*-test) results show (A = 0.01) that sterilization of processed cheese does not cause unequivocal changes in the shape of fat globules. Due to the sterilization treatment, in groups I and IV the values of the shape coefficient decreased, in group III the shape coefficient values increased, and in group II the shape coefficient was not influenced by the sterilization treatment used.

Table 2. Values of shape coefficient t_{ab} for the non-sterilized and sterilized processed cheeses of the four groups under investigation (mean, S.D.)

Processed cheese group	Processed cheese type	Shape coefficient t_{ab}		No. of fat globules measured
		Mean	S.D.	
I	Non-sterilized	1.54	1.62	1671
	Sterilized	0.71	1.64	2179
II	Non-sterilized	1.06	1.73	1875
	Sterilized	0.97	1.70	1515
III	Non-sterilized	1.14	1.64	3564
	Sterilized	1.39	1.67	3353
IV	Non-sterilized	1.03	1.53	2288
	Sterilized	0.67	1.78	2193

Table 3 gives mean values and standard deviations (S.D.) of the fat globule area in individual samples. In the three groups (I, III and IV) of non-sterilized processed cheeses, mean values of fat globule area were significantly lower than mean values in sterilized processed cheeses ($p < 0.01$), which means that in non- sterilized processed cheeses an average size (assessed by area) of fat globules is lower than in sterilized processed cheeses. Only in group II, no significant differences were found.

Table 3. Mean fat globule area for the non-sterilized and sterilized processed cheeses of the four groups under investigation (mean, S.D.)

Processed cheese group	Processed cheese type	Fat globule area (μm²)		No. of fat globules measured
		Mean	S.D.	
I	Non-sterilized	435.79	604.29	1671
	Sterilized	530.59	1139.58	2179
II	Non-sterilized	386.61	680.99	1875
	Sterilized	360.44	369.77	1515
III	Non-sterilized	491.43	536.51	3564
	Sterilized	660.19	1649.48	3353
IV	Non-sterilized	202.92	647.38	2288
	Sterilized	270.17	533.99	2193

The statistical analysis based on the non-parametric Wilcoxon test gave similar results at the same significance level ($p < 0.01$).

In order to find whether further heat sterilization treatment brought up changes in fat globule size (area) distribution in processed cheese samples, we used a comparison of percent occurrence of small fat globules in individual samples. We regarded as small fat globules those objects that did not exceed 500 or 100 μm^2 in area. The data are summarized in Table 4. Fat globule counts were always ascertained for the same area, therefore total fat globule counts in samples with different heat treatments (N and S) could be compared. In the sterilized samples (S), relative counts (%) were always decreased.

Table 4. Comparison of samples – distribution and size of fat globules in the samples

Samples		Total	Up to 100 μm²		Up to 500 μm²	
			No. of globules	%	No. of globules	%
I	N	1671	224	13.41	1251	74.91
	S	2179	102	4.68	1588	72.91
II	N	1875	580	30.95	1457	77.75
	S	1515	299	19.75	1168	77.15
III	N	3564	107	3.00	2463	69.13
	S	3353	260	7.76	2013	60.05
IV	N	2288	705	30.83	2165	94.67
	S	2193	393	17.93	1919	87.55

N: non-sterilized, S: sterilized

Discussion

Developing computer technologies have spread throughout people's lives and are used in most fields of scientific research. At present, the importance and interest in the use of computer controlled image analysis is growing because it facilitates obtaining quantitative characteristics from images and their objective explanation. Computerized image analysis

is a non-destructive method that allows repeated measurements. The advantages include promptness, accuracy and easy sample preparation (Zikmundová 1996).

Final structure of processed cheese is influenced by several factors such as the fat content, pH (Marchesseau et al. 1997), the method of mechanical processing (Klostermeyer and Buchheim 1988) and the emulsifying agents used (Sutheerawattananonda et al. 1997). In this study, each group included samples of the same chemical composition prepared in the same manner (within each group, the melted mass from one batch was divided into two parts). We therefore assume that heat treatment affects the shape of fat particles in processed cheese, however, an unequivocal conclusion regarding a direction of fat globule deformation cannot be drawn.

The size of fat globules in milk is affected by a number of intravital factors. The size of fat particles in milk products depends on technological processing variables and the ingredients used. For instance, Bowland and Foegeding (2001) and Awad et al. (2002) studied the size of fat globules. The size of fat particles was usually assessed depending on some indices of technological process (pH, revolutions per minute, emulsifying agents used) or put in the context of rheological properties of processed cheeses. The basis for determining the size is not always the same: some researchers measured the area and even the diameter of fat globules (Bowland and Foegeding 2001), others the diameter only (Sutheerawattananonda et al. 1997).

In all the samples we investigated, fat globules were divided into groups according to their size. Table 4 shows that there was a higher proportion of small globules (up to 100 μm^2) in the non-sterilized samples, and due to sterilization, a proportion of large fat globules increased. During the sterilization treatment of processed cheese, small fat globules probably merge, resulting in a decrease in their counts and an increase in the area of individual objects.

The statistical analyses showed that in the processed cheese samples under investigation changes in fat globule shape occurred due to heat sterilization. Nevertheless, the data obtained did not unequivocally imply the trend of change in the fat globule shape. However, the heat treatment influenced the size of fat globules. By comparing samples subjected to different heat treatments we found out that after sterilization there were lower counts of small fat globules, which concurrently lead to higher counts of larger fat globules. Therefore, it can be assumed that during further heat treatment of processed cheese the small particles probably merge, thereby increasing their area.

The above-described procedure, based on the computer analysis of microscope images can be used to examine other foods.

Vliv sterilace na velikost a tvar tukových kuliček v modelových tavených sýrech

Modelové vzorky sýrů ze 4 nezávislých výrob byly po procesu tavení a uzavření do obalů následně ošetřeny sterilačním záhřevem (117 °C po dobu 20 minut) s cílem prodloužit dobu jejich trvanlivosti. Cílem práce byla aplikace metody obrazové analýzy na posouzení změny velikosti a tvaru tukových kuliček vlivem sterilačního záhřevu. Součástí práce byl také výběr vhodného postupu pro přípravu mikroskopických preparátů, zhotovení mikrofotografií a šablony pro automatické zpracování snímků obrazovým analyzátorem, zjištění parametrů pro určení velikosti a tvaru tukových kuliček a statistické zpracování výsledků.

Na základě provedeného experimentu lze dojít k názoru, že změna tvaru tukových kuliček v důsledku sterilačního záhřevu není jednoznačná. Na druhou stranu bylo zjištěno, že vlivem termosterilačního ošetření (117 °C po dobu 20 minut) se velikost tukových kuliček signifikantně zvýšila ($p < 0,01$), což bylo doprovázeno snížením podílu menších tukových kuliček (do 500 μm^2, resp. do 100 μm^2) ve vzorcích sterilovaných tavených sýrů. Z výsled-

ků rovněž vyplývá, že metoda analýzy obrazu je plně využitelná při posuzování vlivu technologického procesu na jakost tavených sýrů.

Acknowledgments

The present study was supported by the Ministry of Education, Youth and Sports, the Research Project No. 6215712402 (Veterinary aspects of food safety and quality).

References

AGRESTI A 1984 Analysis of ordinal categorical data. John Wiley & Sons, Inc.USA, 287 p.

AWAD RA, ABDEL-HAMID LB, EL-SHABRAWY SA, SINGH RK 2002: Texture and microstructure of block type processed cheese with formulated emulsifying salt mixtures. Lebensm - Wissensch Technol **35**: 54-61

BIJKER PGH, KOOLMEES PA, van LOGTESTIJN JG 1983 Tissue composition of mechanically deboned pork (MDP). Meat Sci **9**: 257- 269

BOWLAND EL, FOEGEDING EA 2001 Small strain oscillatory shear and microstructural analysis of a model processed cheese. J Dairy Sci **84**: 2372-2380

BYLUND G 1995: Dairy Processing Handbook. Lund: Tetra Pak Processing Systems, 436 p.

CACCAMO M, MELILLI C, BARBANO D M, PORTELLI G, MARINO G, LICITRA G 2004: Measurement of gas holes and mechanical openness in cheese by image analysis. J Dairy Sci **87**: 739-748

CARIĆ M, KALÁB M 1997: Processed cheese products. In Fox, P.F. (Ed.) Cheese: Chemistry, Physics and Microbiology. Volume 2. Major Cheese Groups, 2. ed. Elsevier Applied Science, London and New York, pp. 467-505

GUINEE TP 2003 Pasteurized processed cheese products. In Roginski, H., Fuquay, J.W., Fox, P.F. (Eds) Encyclopedia of Dairy Science, Volume 1. London: Elsevier Science, pp. 411-418

HEERTJE I, LEUNIS M 1997 Measurement of shape and size of fat crystals by electron microscopy. Lebensm - Wissensch Technol **30**: 141-146

HILDEBRANDT G, HIRST L 1985 Determination of the collagen, elastin and bone content in meat products using television image analysis. J Food Sci **50**: 568-570

HOLCOMB DN, PECHAK DG, CHAKRABATI S, OPSAHL A 1992: Visualizing textural changes in dairy products by image analysis. Food Technol **46**: 122-127

KLOSTERMEYER H, BUCHHEIM, W 1988: Die Mikrostruktur von Schmelzkäseerzeugnissen. Kieler Milchwirtschaftliche Forschungsberichte **40**: 219-231

MAFART P, COUVERT O, LEGUÉRINEL I 2001: Effect of pH on the heat resistance of spores. Comparison of two models. Int J Food Microbiol **63**: 51-56

MARCHESSEAU S, GASTALDI E, LAGAUDE A, CUQ J 1997: Influence of pH on protein interactions and microstructure of process cheese. J Dairy Sci **80**: 1483-1489

PALKA K, DAUN H 1999 Changes in texture, cooking losses and myofibrilar structure of bovine *M. semitendinosus* during heating. Meat Sci **51**: 237-243

RAVAL DM, MISTRY VV 1999: Application of ultrafiltered sweet buttermilk in the manufacture of reduced fat process cheese. J Dairy Sci **82**: 2334-2343

SUTHEERAWATTANANONDA M, FULCHER RG, MARTIN FB, BASTIAN ED 1997 Fluorescence image analysis of process cheese manufactured with trisodium citrate and sodium chloride. J Dairy Sci **80**: 620-627

STANDARDISATION AGREEMENT (STANAG) 2937 2001: Survival, Emergency and Individual Combat Ration - Nutritional Values and Packaging. 3. ed. Brussels: NATO/MAS

TREMLOVÁ B, ŠTARHA P 2002: Histometric evaluation of meat products - determination of size and number of objects. Czech J Food Sci **20**: 175-180

YOON WB, McCARTHY KL 2003: Flow behavior of processed chesee melts. J Food Process Engineering **26**: 559-576

WANG HH, SUN DW 2002 Melting characteristic of cheese: analysis of effects of cooking conditions using computer vision technology. J Food Engineering **51**: 305-310

WANG HH, SUN DW 2003: Assessment of cheese browning affected by baking conditions using computer vision. J Food Engineering **56**: 339-345

ZIKMUNDOVÁ M 1996: The use of image analysis for food quality evaluation. Master Thesis. Institute of Chemical Technology, Prague, 98 p. (in Czech)

The Effect of Lipolytic Enzymes of *Bacillus* spp. on Quality of Ultra-High-Temperature-Treated Milk

B. JANŠTOVÁ, L. VORLOVÁ, M. DRAČKOVÁ

Department of Milk Hygiene and Technology, Faculty of Veterinary Hygiene and Ecology
University of Veterinary and Pharmaceutical Sciences Brno

Received June 7, 2005
Accepted June 30, 2006

Abstract

Janštová B., L. Vorlová, M. Dračková: The Effect of Lipolytic Enzymes of *Bacillus* spp. on Quality of UHT Milk. Acta Vet Brno 2006, 75: 427-435.

Lipolysis was monitored based on determining the concentration of free fatty acids in milk, on the model case of UHT milk contamination with spores of 15 *B. licheniformis*, *B. subtilis* and *B. cereus* strains isolated from farm environment and raw milk. Lipolysis was not recorded at storage temperature of 4 °C, whereas significant changes in levels of free fatty acids were shown at storage temperature of 24 °C. After 3 weeks of storage the initial content of 41.97 mmol·kg^{-1} of fat rose to as much as 1,617.22 mmol·kg^{-1} of fat. The extent of the change depended mainly on the *Bacillus* spp. species and the storage period and, to a certain degree, also on the initial number of microorganisms. Significant lipolytic activity was detected in association with *B. licheniformis* and *B. cereus* species. It was found that spores of resistant *B. licheniformis* strains may survive 100 °C/10 min and 135 °C/5 s heating and show lipolytic activity.

Lipolysis, spores, Bacillus spp., free fatty acids, milk

Bacillus spp. microorganisms represent important contaminants of raw milk from the point of view of both hygiene and technology, and in some cases, human health.

Bacillus spp. spores are commonly present in stables, causing mainly secondary milk contamination during milking. A number of representatives of the *Bacillus* family are present in milk as psychrotrophic microflora. The species of the *Bacillus* family most frequently isolated from raw milk are *B. licheniformis* and *B. cereus* (Crielly et al. 1994). Páčová et al. (1996) report that the species with the widest distribution is *B. licheniformis*. The distribution of other species is lower (Lukášová et al. 2001; Vyletělová et al. 2001). Spores of thermostable bacteria may pollute a product even during the technological process, and they may be present in UHT milk, too, as reported e.g. by Bahout (2000), who detected the spores at concentrations of up to 2.6×10^2·ml^{-1} in 18.3% of tested samples.

Bacillus spp. microorganisms are an extremely burdensome part of raw milk microflora, as removing their spores is very difficult due to their thermoresistance. The spores may be partly damaged by pasteurization, but mostly cannot survive sterilization and the UHT process. One important characteristic of *Bacillus* spp. is the ability of the vegetative cells to produce thermostable extracellular enzymes after proliferation (Meer et al. 1991; Ipsen et al. 2000). The proteolytic and lipolytic activity of these enzymes bears on nutritional and sensory properties of milk products even when no live bacteria are present (Boor et al. 1998). Brown (2000) refers to *Bacillus* spp. as microorganisms causing significant economic loss.

Some *Bacillus* spp. species, especially *B. cereus*, show a high degree of lipolytic and proteolytic activity (Janštová et al. 2004). The enzyme activity is demonstrated in a number of milk and milk-product defects, such as those described by Silveira et al. (1999). Lipolytic enzymes produced by *Bacillus* spp. microorganisms cause fat hydrolysis

Address for correspondence:
MVDr. Bohumíra Janštová, Ph.D.
Department of Milk Hygiene and Processing
University of Veterinary and Pharmaceutical Sciences
1/3 Palackého St., 612 42 Brno, Czech Republic

Phone: +420 541 562 712
Fax: +420 541 562 711
E-mail: janstovab@vfu.cz
http://www.vfu.cz/acta-vet/actavet.htm

and production of free fatty acids after germination of spores in milk and milk products. Microbial enzymes are strictly specific and much more active than native lipases (Murray et al. 2001). Johnston and Bruce (1982), who studied lipolytic properties of *Bacillus* spp., detected fat lipolysis in 52% of strains and a 77% prevalence of lecithinase-positive strains.

Since lipases are largely produced by microorganisms in the late lag stage and the early stationary growth stage, there is no direct proportional correlation between the number of microorganisms and the enzyme concentration. Defects are detected when the concentration of microorganisms reaches 5×10^5 - 10^7 CFU·ml^{-1} (Vyletělová et al. 2000; Marth and Steele 1998).

Unlike milk lipases, microbial lipases are thermoresistant and remain active despite heating including UHT milk treatment, being able to cause the development of rancid taste and flavour, contributing thus to product degradation. Lipase activity thus presents an obstacle to successful implementation of UHT products where lipases are contained in the raw material, and their activity in the UHT product is preserved.

It is low-carbon fatty acids (C_4 - C_{12}), especially the butyric acid, that contribute to the development of sensory defects the most. Fatty acids C_4 - C_8 are to blame for the rancid flavour while the foul, bitter and soapy flavour is due to C_{10} - C_{12} fatty acids (Champagne et al. 1994).

Lipase activity is enhanced by physical phenomena applied in milk processing such as homogenisation, sudden temperature change, intensive stirring or milk turbulence in the pipes, as they may damage the lipoprotein membrane of fat globules, making the fat vulnerable to lipase activity.

Materials and Methods

The experiments were conducted on collection strains of the Department of Milk Hygiene and Processing of the University of Veterinary and Pharmaceutical Sciences Brno. A total of 15 *Bacillus* spp. strains isolated from farm environment and raw milk were used. Each experiment involved 5 *B. licheniformis*, 5 *B. subtilis* and 5 *B. cereus* strains. All the isolated strains were identified by Lukášová et al. (2001). Durable UHT milk distributed on the market was used as the medium for *Bacillus* spp. spore contamination. The UHT milk originated from the same batch.

Milk was inoculated with *Bacillus* spp. spore suspensions, without using thermic inactivation, in such an amount as to reach spore concentrations of 10^2, 10^1, $< 10^1$ in 1 ml of milk. Besides that, *Bacillus* spp. spore suspensions heated to 100 °C in water bath (exposure time 10 min) and to 135 °C in glycerol bath (exposure time 5 s) were used.

Thermoinactivation of spore suspension (5 ml) was performed after heating to 70 °C. The complete inactivation of *B. subtilis* and *B. cereus* spores was performed after heating, at 100 °C for 10 min and 135 °C for 5 s. The initial concentration of *B. licheniformis* thermoresistant strain was reduced by 1 logarithmic order.

Milk samples were stored in closed sterile glass sample bottles at 24 °C in a thermostat and at 4 °C in a refrigerator.

The proof and quantification of lipolysis due to *Bacillus* spp. microorganisms was based on the extraction/titration method for determining substance content of free fatty acids (FFA) in accordance with ČSN 57 0533 "Determination of Substance Content of Free Fatty Acids" (1997). The amount of substance for FFA was expressed in mmol·kg^{-1} of milk fat. The analysis was performed at 1-week intervals for 3 weeks. All samples were analyzed in triplicate.

To be able to compare the dynamism of growth of the microorganisms and change the values of observed indicators, we performed a microbiological analysis of milk samples, namely determination of the total number of spore-forming microorganisms (ČSN ISO 6887, 1994). Cultivation was performed on Plate Count Agar (HiMedia, India). Sensory evaluation (colour, coagulum, and odour) was performed, too, when the samples were taken for analysis.

The STAT Plus Statistical and Graphic System (Matoušková et al. 1992) was used to assess the significance of variances of individual indicators at levels $p < 0.01$ and $p < 0.05$. The data processing was based on dispersion analysis and Scheffe's contrasts the Turkey's test of variance significance. To obtain correct results, the data were subjected to the Box-Cox transformation prior to variance analysis so as to ensure that they meet the condition of basic distribution normality. Before the analysis proper, homogeneity of variances of the tested samples had to be verified. The Bartlett's test was used for this purpose.

Results and Discussion

Microorganisms of the *Bacillus* family are important producers of lipolytic enzymes. Thermoresistant bacterial lipases may represent a serious obstacle to UHT processing of milk products containing fat.

No increase in FFA content due to any of the considered *Bacillus* spp. species compared with the initial FFA levels occurred during sample storage at 4 °C; the detected levels were consistent with control samples (the results are not mentioned). None of the considered strains proliferated at 4 °C. Our results achieved by FFA content determination in samples stored at this temperature are in line with the conclusions of Burdová (2002), who stressed the importance of storage temperature based on her finding that milk storage temperature of 4 °C was not high enough to ensure production of an amount of lipolytic and proteolytic enzymes comparable to that produced in samples stored at 10 °C for 2 - 3 days despite the storage period being three times longer.

An increase in FFA caused by lipolytic enzyme activity was recorded only in association with sample storage at 24 °C and correlated with the increase in the number of microorganisms. Some change was detected already when levels 10^4 - 10^5 CFU·ml^{-1} were achieved; CFU peaked in the 3rd week of storage and was identical for all *Bacillus* spp. thermally non-inactivated strains, namely 10^8·ml^{-1} of milk. A lower number of CFU was found when we used non-inactivated *B. licheniformis* spores. *B. subtilis* and *B. cereus* were completely inactivated. No germination or growth was observed. The dynamics of changes of *B. licheniformis* number depending on time storage is demonstrated in Fig. 1. No increase of FFT content was found in control samples during the experiment.

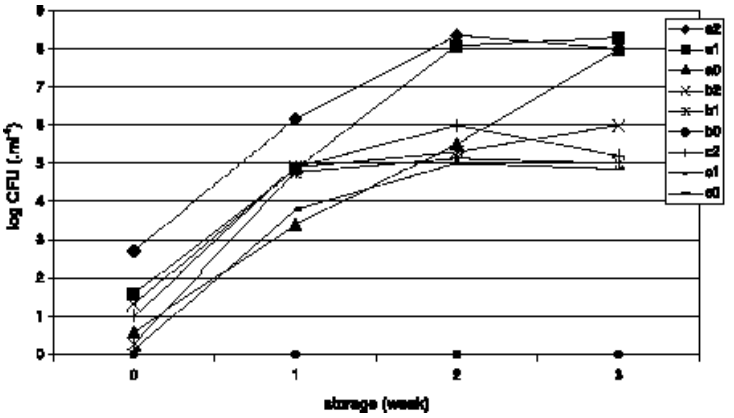

Fig. 1. Dynamics of changes of CFU number in milk containing *B. licheniformis* spores stored at 24 °C depending on inactivation method (a - no inactivation, b - inactivation by heating to 100 °C/10 min, c - inactivation by heating to 135 °C/5 s) and initial spore concentration (2 = 10^2 in 1 ml, 1 = 10^1 in 1 ml, 0 = < 10^1 in 1 ml).

Significant lipolysis was detected in samples with higher initial content of inoculated, thermally non-inactivated spores in milk; small-scale lipolysis was detected in samples containing few individual spores. The average initial FFA content in the milk used for the experiments was 41.97 mmol·kg^{-1} of milk fat.

As shown in Table 1, the use of the highest concentration of thermally non-inactivated and a 3-week storage period led to an increase of FFA content in milk in relevant *B. licheniformis* strains depending on the initial concentration. The initial number of spores being 10^2·ml^{-1}, the average content was 330.53 ± 80.98; 940.74 ± 482.77; and 1,129.61 ± 387.42 mmol·kg^{-1} of

Table 1. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing non-inactivated *B.licheniformis, B. subtilis, B. cereus* spores. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		10^2 ± SD min max	10^1 ± SD min max	< 10^1 ± SD min max
Initial FFA content 41.97				
B. licheniformis	1	330.53 ± 80.98	255.89 ± 138.79	212.12 ± 131.73
		239.68	106.29	58.81
		443.60	446.4	353.21
	2	940.74 ± 482.77	758.80 ± 389.69	459.22 ± 294.36
		417.10	329.29	147.61
		1513.40	1265.90	942.75
	3	1129.61 ± 387.42	1104.82 ± 366.18	787.68± 144.75
		598.40	679.29	565.20
		1665.40	1504.40	1109.80
B. subtilis	1	145.16 ± 70.26	124.02 ± 97.89	79.59 ± 21.77
		54.67	42.94	42.98
		219.44	294.55	95.50
	2	467.78 ± 161.78	227.74 ± 89.04	203.24 ± 38.89
		118.32	86.99	43.25
		515.92	332.96	138.25
	3	488.61 ± 67.67	408.59 ± 53.57	354.22 ± 18.54
		280.60	191.52	43.11
		536.46	617.75	582.16
B. cereus	1	241.79 ± 160.48	202.63 ± 129.53	157.71 ± 147.84
		62.24	67.13	64.69
		412.35	382.80	364.72
	2	771.44 ± 485.22	838.98 ± 559.94	701.96 ± 438.31
		130.59	118.60	110.41
		1240.70	1381.00	1109.80
	3	1617.2 ± 68.17	1261.1 ± 31.11	895.0 ± 13.01
		1502.55	1068.25	885.60
		1665.40	1425.50	904.40

fat, after 1, 2 and 3 weeks, respectively. The maximum level recorded for the individual strains was 1,665.22 mmol·kg^{-1} of fat. The initial content of 10^1·ml^{-1} led to similarly high levels. The initial spore concentration of < 10^1·ml^{-1} produced a high determined amount of FFA, too (787.68 ± 144.75 mmol·kg^{-1} of fat).

The number of microorganisms in milk being roughly the same, lipolytic activity of *B. subtilis* enzymes was the least significant compared with other considered bacterial strains. At spore concentration of 10^2 per 1 ml of milk, the average FFA levels were 467.78 ± 161.78 mmol·kg^{-1} of fat and 488.61 ± 67.67 mmol·kg^{-1} of fat after 2 and 3 weeks of storage respectively. The initial spore concentration had a significant differentiating effect on FFA milk content: FFA content was significantly lower at spore content < 10^1 per 1 ml of milk and no FFA content increase at all occurred in 2 of the 5 tested strains.

The *B. cereus* strain was generally characterized by strong lipolytic activity, to which the high FFA levels found even in samples with the lowest initial spore content testify. The values for the individual strains however varied considerably. With the initial number of spores 10 ^{2} per ml^{-1}, the determined levels of FFA were 241.79 ± 160.48, 771.44 ± 485.22

and 1617.22 ± 68.17 mmol·kg^{-1} of fat. The final FFA content levels at lower spore concentrations were 1,261.1 ± 31.11 and 895.00 ± 13.01 mmol·kg^{-1} of fat.

Significant variances ($p < 0.05$) and highly significant variances ($p < 0.01$) were found between the activities of individual *Bacillus* spp. species tested. FFA content for *B. subtilis* was lower ($p < 0.05$) compared with *B. licheniformis* and lower ($p < 0.01$) compared with *B. cereus*.

When spores inactivated by heating to 100 °C for 10 min were used in samples stored at 24 °C, lipolysis was observed only in the *B. licheniformis* thermoresistant strain. Thermoresistance of *Bacillus* spp. spores was described by Janštová and Lukášová (2001). At higher initial concentrations of inoculated spores, the survivor spores of the strain germinated and FFA content increased to 206.36, 417.80 and 605.21 mmol·kg^{-1} of fat (after 1st, 2nd and 3rd week of storage). The average FFA content levels after 3 weeks of storage were 230.33 ± 324.47 mmol·kg^{-1} (initial concentration of spores was 10^2·ml^{-1}) and 144.25 ± 6.26 mmol·kg^{-1} of fat (initial concentration of spores was 10^1·ml^{-1}) (see Table 2a). When spores of other *B. licheniformis* strains and also *B. subtilis* and *B. cereus* spores were heated, they were eradicated and no lipolysis was recorded (see Table 2b).

Table 2a. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing *B. licheniformis* spores inactivated by heating to 100 °C/10 min. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		10^2 ± SD min max	10^1 ± SD min max	< 10^1 ± SD min max
Initial FFA content 41.97				
B. licheniformis	1	97.19 ± 95.55	84.12 ± 0.28	42.11 ± 0.16
		42.05	41.59	42.00
		206.36	70.18	42.68
	2	168.06 ± 216.29	112.11 ± 0.31	43.02 ± 0.22
		42.09	42.06	42.42
		417.80	72.56	45.02
	3	230.33 ± 324.47	144.25 ± 6.26	43.38 ± 0.08
		44.28	42.06	42.11
		603.25	344.72	45.02

Table 2b. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing *B. subtilis* and *B. cereus* spores inactivated by heating to 100 °C/10 min. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		10^2 ± SD	10^1 ± SD	< 10^1 ± SD
Initial FFA content 41.97				
B. subtilis	1	42.35 ± 0.49	42.85 ± 1.37	42.52 ± 0.57
	2	43.11 ± 1.51	42.63 ± 1.16	42.88 ± 1.12
	3	43.05 ± 2.04	42.54 ± 1.12	42.96 ± 2.08
B. cereus	1	42.58 ± 0.38	43.25 ± 0.71	43.08 ± 0.35
	2	42.62 ± 0.71	42.15 ± 1.07	42.42 ± 1.68
	3	42.55 ± 2.11	42.32 ± 0.98	42.60 ± 1.25

When inactivated by heating to 135 °C for 5 s (see Table 3a), a limited amount of the same *B. licheniformis* thermoresistant strain germinated. With the initial spore concentrations 10^1 and 10^2·ml^{-1}, the spores germinated to reach 10^5 - 10^6 CFU·ml^{-1} of milk. Lipolytic activity

Table 3a. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing *B. licheniformis* spores inactivated by heating to 135 °C/5 s. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		$10^2 \pm$ SD min max	$10^1 \pm$ SD min max	$< 10^1 \pm$ SD min max
Initial FFA content 41.97				
B. licheniformis	1	100.42 ± 82.26	100.08 ± 81.89	42.37 ± 0.16
		42.25	42.17	42.25
		158.58	157.98	42.48
	2	302.74 ± 367.82	199.40 ± 221.49	42.78 ± 0.31
		42.65	42.78	42.41
		562.83	356.02	43.15
	3	537.32 ± 699.01	473.76 ± 556.01	42.80 ± 0.30
		43.04	43.12	42.24
		1031.60	904.40	43.41

was subsequently demonstrated as an increase in average FFA levels was to 537.32 ± 699.01 (initial concentration of spores was 10^2 ml^{-1}) 473.76 ± 556.64 mmol·kg^{-1} (initial concentration of spores was 10^1·ml^{-1}) of fat in the 3 rd week of storage. No increase in FFA content was recorded in samples with the lowest initial inactivated spore concentrations. Spores of other *B. licheniformis* strains and *B. subtilis* and *B. cereus* spores were completely inactivated; no lipolysis was recorded (see Table 3a, Table 3b).

Table 3b. Average FFA content levels (mmol·kg^{-1} of milk fat) in milk containing *B. subtilis, B. cereus* spores inactivated by heating to 135 °C/5 s. Dependence on storage period (week) at 24 °C

Species	Week	Initial No. of spores/1 ml of milk		
		$10^2 \pm$ SD	$10^1 \pm$ SD	$< 10^1 \pm$ SD
Initial FFA content 41.97				
B. subtilis	1	42.85 ± 0.50	42.25 ± 0.08	42.68 ± 0.59
	2	43.14 ± 0.86	43.91 ± 2.11	42.68 ± 0.69
	3	43.32 ± 0.40	43.24 ± 0.25	42.78 ± 0.83
B. cereus	1	43.08 ± 0.85	42.38 ± 0.99	42.73 ± 0.24
	2	43.32 ± 0.08	43.03 ± 0.33	43.07 ± 0.38
	3	43.29 ± 0.20	43.04 ± 0.05	43.09 ± 0.03

The dynamics of FFA content growth depending on thermo-inactivation mode and initial *B. licheniformis* spore concentration is shown in Fig. 2.

The significance of the variances between FFA levels detected in samples inoculated with thermoresistant *B. licheniformis* strain spores inactivated by heating to 100 °C for 10 min and to 135 °C for 5 s on the one hand and the other strains of the same species on the other is highly significant ($p < 0.01$). Just as highly significant variances ($p < 0.01$) were found when average FFA content levels in samples with *B. licheniformis* inoculation were compared with those of other tested *Bacillus* spp. species.

Our experiments have proved that thermo-inactivation of spores, depending on the temperature, causes reduction or cessation of lipase production as a consequence of sublethal or lethal spore damage. However, if the strain is a thermoresistant one or if

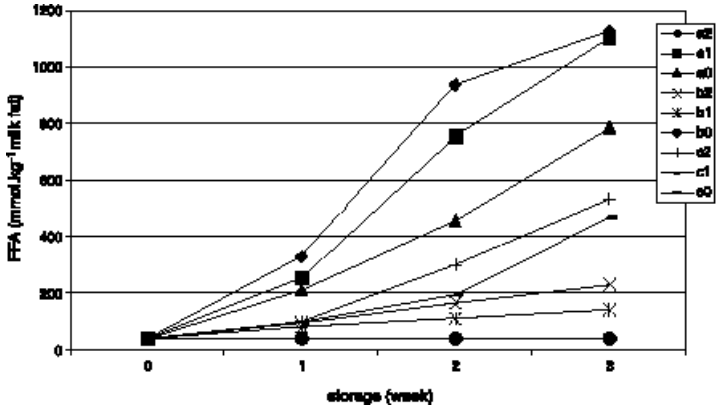

Fig. 2. FFA content in milk containing *B. licheniformis* spores stored at 24 °C depending on inactivation method (a - no inactivation, b - inactivation by heating to 100 °C/10 min, c - inactivation by heating to 135 °C/5 s) and initial spore concentration (2 = 10^2 in l ml, 1 = 10^1 in 1 ml, 0 = < 10^1 in 1 ml)

enzymes are produced before thermic treatment of milk, the spores remain active or partly active even in the finished product. Grieger et al. (1990) report that heating milk above 130 °C for 5 - 10 min leads to reduction of lipases to 10% and that inactivation of some lipases may require temperatures as high as 150 °C.

Characteristics of microbial lipases and their role in food spoilage was studied e.g. by Stead (1986). In consistence with our findings, Vlachos and Litopoulou-Tzanetaki (1985) report differences as to production of lipolytic enzymes between individual *Bacillus* spp. species including between strains of the same species. They found no proofs of lipolytic activity of some *B. licheniformis* strains.

Despite the fact that due to differences of focus and use of different determination methods our findings could not be compared with data published by other authors, it is clear that the process of lipolysis can be recorded by the method we used. In contrast to the requirement of ČSN 570529 (1993) - requiring that the FFA content in raw milk for dairy technologies and processing determined by the titration/extraction method does not exceed 32 mmol·kg^{-1} - a mild increase in FFA content in UHT milk and a subsequent growth due to lipolysis caused by *Bacillus* spp. enzymes was observed.

Action of bacterial enzymes produced by microorganisms including some microorganisms from the *Bacillus* family is the cause of sensory defects of milk and milk products. Enzymes isolated from *B. licheniformis, B. coagulans* and *B. subtilis* were studied by Kalogridou-Vassiliadou (1992), reporting an association with milk spoilage. Matta and Punj (1999) speak of a connection between flavour defects and the 48% prevalence of lipolytic bacillary strains in raw milk samples they detected. Defects originate upon reaching a certain concentration of microorganisms; sensory alterations of milk can be detected at concentrations of 10^5 - 10^7 CFU·ml^{-1} (Vyletělová et al. 2000; Marth and Steele 1998; Šilhánková 1999). In contrast to that, the content of psychrotrophic CFU sufficient to initiate lipolysis reported by Silveira et al. (1999) is 2.7×10^4 CFU·ml^{-1}.

No sensory alterations were recorded in samples stored at 4 °C, despite 3 months of storage, in association with any of the *Bacillus* species. Deterioration of sensory qualities of milk (bad odour, running) was recorded in samples containing non-inactivated spores stored at 24 °C in correspondence to the process of lipolysis and proteolysis. No sensory change was detected in thermically inactivated samples, with the exception of milk samples inoculated with *B. licheniformis* thermoresistant strain spores. In consistence with our

results, milk defects such as running, gelation, ropiness, colour and flavour defects, fat clotting, and cream bitterness are described by Te Giffel et al. (1996), Bassette et al. (1986), Walstra et al. (1999). Cream bitterness is the most widely spread defect caused by *B. cereus*, the cause being fat destabilization due to phospholipase C, degrading fat globule membranes by enzymatic processes (Al-Kanhal 1985; Meer et al. 1991). Lecithinase, too, causes cream flocculation; the process involves aggregation of fat globules after their envelopes are destructed (Harrigan 1998).

Vliv lipolytických enzymů Bacillus spp. na kvalitu UHT mléka

Sledování lipolýzy bylo provedeno na základě stanovení látkového obsahu volných mastných kyselin v mléce a to na modelovém případě kontaminace trvanlivého mléka sporami 15 kmenů *B. licheniformis, B. subtilis* a *B. cereus* izolovaných z prostředí farmy a ze syrového mléka. Při skladovací teplotě 4 °C nebyla lipolýza zaznamenána, zatímco při skladovací teplotě 24 °C byly zjišťovány výrazné změny obsahu volných mastných kyselin. Po 3 týdnech skladování došlo ke zvýšení obsahu volných mastných kyselin z počáteční hodnoty 41.97 mmol.kg^{-1} tuku na hodnotu až 1617.22 mmol·kg^{-1} tuku. Rozsah změn závisel především na druhu *Bacillus* spp. a době skladování a v určité míře také na výchozím počtu mikroorganismů. Výrazná lipolytická aktivita byla zjištěna u druhů *B. licheniformis* a *B. cereus*. Bylo zjištěno, že teplotní a časový parametr záhřevu na 100 °C 10 min a 135 °C 5 s mohou spory rezistentních kmenů *B. licheniformis* přežít a vykazovat lipolytickou aktivitu.

Acknowledgements

The study was supported from the grant provided by the Ministry of Education, Youth and Sports of the Czech Republic No. MSM 6215712402.

References

AL-KANHAL HA, FRANK JF, CHRISTEN GL 1985: Microbial protease and phospholipase C stimulate lipolysis of washed cream. In GRIFFITHS MW 1992: *B. cereus* in milk and milk products. Bulletin FIL-IDF **275**: 37. J Dairy Sci **68**: 3162-6170

BAHOUT AA 2000: Prevalence of *Bacillus* species in UHT milk. Assiut Vet Med J **42**: 47-53

BASSETTE R, FUNG DY, MANTHA VR 1986: Off - flavors in milk. Crit Rev Food Sci Nutr **24**: 1-52

BOOR KJ, BROWN DP, MURPHY SC, KOZLOWSKI SM, BANDLER DK 1998: Microbiological and chemical quality of raw milk in New York State. J Dairy Sci **81**: 1743-1748

BROWN KL 2000: Control of bacterial spores. Brit Med Bull **56**: 158-171

BURDOVÁ O, BARANOVÁ M, LAUKOVÁ A, ROZANSKÁ H, ROLA JG 2002: Hygiene of pasteurised milk depending on psychrotrophic microorganisms. B Vet I Pulawy **46**: 325-329

CHAMPAGNE CP, LAING RR, ROY D, MAFU AA, GRIFFITHS MW 1994: Psychrotrophs in dairy products: Their effects and their control. Crit Rev Food Sci Nutr **34**:1-30

CRIELLY EM, LOGAN NA, ANDERTON A 1994: Studies on the *Bacillus* flora of milk and milk products. J Appl Bacteriol **77**: 256-263

ČSN ISO 6887 1994: Mikrobiologie. Všeobecné pokyny pro přípravu ředění při mikrobiologickém zkoušení. Český normalizační institut, Praha, 7 p.

ČSN 57 0529 1993: Syrové kravské mléko pro mlékárenské ošetření a zpracování. Český normalizační institut, Praha, 8 p.

ČSN 57 0533 1997: Stanovení látkového obsahu volných mastných kyselin. Praha, Český normalizační institut, p. 8.

GRIEGER C, HOLEC J et al. 1990: Hygiena mlieka a mliečnych výrobkov. Bratislava, Príroda, pp. 39-40.

HARRIGAN WF 1998: Laboratory Methods in Food Microbiology. Academic Press. London, pp. 202-245.

IPSEN R, OTTE J, LOMHOLT SB, QVIST KB 2000: Standardized reaction times used to describe the mechanism of enzyme-induced gelation in whey protein systems. J Dairy Res **37**: 403-413

JANŠTOVÁ B, LUKÁŠOVÁ J 2001: Heat resistance of *Bacillus* spp. spores isolated from cow's milk and farm environment. Acta Vet Brno **70**: 179-184

JANŠTOVÁ B, LUKÁŠOVÁ J, DRAČKOVÁ M, VORLOVÁ L 2004: Influence of *Bacillus* spp. Enzymes on UHT Milk Proteins. Acta Vet Brno **73:** 393-400

JOHNSTON DW, BRUCE J 1982: Incidence of thermoduric psychrotrophs in milk produced in the West of Scotland. J Appl Bacteriol **53**: 333-337

KALOGRIDOU-VASSILIADOU D 1992: Biochemical activities of *Bacillus* species isolated from flat sour evapored milk. J Dairy Sci **75**: 2684-2686.
LUKÁŠOVÁ J, VYHNÁLKOVÁ J, PÁČOVÁ Z 2001: *Bacillus* species in raw milk and in the farm environment. Milchwissenschaft **56**: 609-611
MARTH EH, STEELE JL 1998: Applied Dairy Microbiology. Marcel Decker, INC, New York, p. 516.
MATOUŠKOVÁ O, CHALUPA J, CÍGLER M, HRUŠKA K 1992: STAT-Plus uživatelská příručka, verse 1.01. Veterinary Research Institute, Brno, CR, p. 168.
MATTA H, PUNJ V 1999: Isolation and identification of lipolytic, psychrotrophic spore forming bacteria from raw milk. Int J Dairy Technol **52**: 59-62
MEER RR, BAKER FW, BODYFELT FW, GRIFFITHS MW 1991: Psychrotrophic *Bacillus* spp. in fluid milk products. A review. J Food Protect **54**: 969-979
MURRAY RK, GRANNER DK, MAYES PA, RODWELL VW 2001. Harperova biochemie. H a H Vyšehradská s.r.o., p. 872.
PÁČOVÁ Z, VYHNÁLKOVÁ J, LUKÁŠOVÁ J, HOLEC J 1996: Identification of aerobic and facultatively anaerobic sporulating bacteria isolated from operations of milk primary production. Ved Med-Czech **41**: 19-23
SILVEIRA IA, CARVALHO EP, TEIXEIRA D, BARRIOS BE 1999: Verification of the proteolytic and lipolytic activities of the microbial flora isolated from raw, refrigerated, type B milk. II. Psychrotrophic microorganisms. Rev Latinoam Microbiol **41**: 85-89
STEAD D 1986: Microbial lipases: their characteristic, role in food spoilage and industrial uses. J Dairy Res **53**: 481-505
ŠILHÁNKOVÁ L. 1999: Mikrobiologie pro potravináře a biotechnology. Victoria publishing a.s., Praha, p. 304.
TE GIFFEL MC, BEUMER RR, BONESTROO MH, ROMBOUTS FM 1996: Incidence and characterization of *Bacillus cereus* in two dairy processing plants. Neth Milk Dairy J **50**: 472-492
VLACHOS I, LITOPOULOU-TZANETAKI E 1985: Free fatty acid production by some *Bacillus* strains grown in UHT milk at room temperature. Milchwissenschaft **40**: 521
VYLETĚLOVÁ M, HANUŠ O, URBANOVÁ E, KOPUNECZ P 2000: The occurrence and identification of psychrotrophic bacteria with proteolytic and lipolytic activity in bulk milk samples at storage in primary production conditions. Czech J Anim Sci **45**: 373-383
VYLETĚLOVÁ M, HANUŠ O, PÁČOVÁ Z, ROUBAL P, KOPUNECZ P 2001: Frequency of *Bacillus* bacteria in raw cow's milk and relation to other hygiene parameters. Czech J Anim Sci **46**: 260-267
WALSTRA P, GEURTS TJ, NOOMEN A, JELLEMA A, BOEKEL MAJS. 1999: Dairy Technology. Principles of Milk Properties and Processes. Marcel Dekker, INC, New York, p. 727.

ACTA VET. BRNO 2006, 75: 437-448; doi:10.2754/avb200675030437

Stress Response to Long Distance Transportation of Common Carp (*Cyprinus carpio* L.)

R. DOBŠÍKOVÁ[1], Z. SVOBODOVÁ[1,2], J. BLAHOVÁ[1], H. MODRÁ[1], J. VELÍŠEK[2]

[1]University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic
[2]University of South Bohemia České Budějovice, Czech Republic

Received October 27, 2006
Accepted April 13, 2006

Abstract

Dobšíková R., Z. Svobodová, J. Blahová, H. Modrá, J. Velíšek: Stress Response to Long Distance Transportation of Common Carp (*Cyprinus carpio* L.). Acta Vet Brno 2006, 75: 437-448.

The stress responses and changes in biochemical and haematological indices were investigated in three-year-old common carp (*Cyprinus carpio* L.) during a long-distance transportation in special truck tanks. Twelve-hour transportation caused a significant increase in ammonia ($p < 0.01$), mean corpuscular volume MCV ($p < 0.01$), metamyelocytes ($p < 0.05$) and band neutrophils ($p < 0.01$), and a significant decrease in Cl^- ($p < 0.05$), lactate ($p < 0.05$), ALT ($p < 0.05$) and ALP ($p < 0.01$) levels. The values of LDH ($p < 0.01$), AST ($p < 0.05$), CK ($p < 0.01$) and haematocrit PCV ($p < 0.05$) were also significantly influenced by the transportation, but no time-dependent relation was found. On the contrary, the levels of cortisol, glucose and total protein in the biochemical profile, and the values of erythrocyte count (RBC), haemoglobin (Hb), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC), leukocyte counts (WBC) and leukogram (except for metamyelocytes and band neutrophils) in the haematological profile were not significantly influenced by the transportation. Results showed that pre-transport fish manipulation (hauling, netting, handling, loading) was found to be an important stressor for fish. Long-distance transportation itself was relatively considerate for the common carp tested.

Fish, transport, haematological parameters, biochemical parameters

In food animal industries, handling and transport are often stressful events. In fish aquaculture, the processes of grading, capturing (most commonly by netting), transferring out of water and transporting fish are inherently stressful, and lead to a "fight or flight" stress response (Barton et al. 1980). This response causes the primary release of adrenalin and cortisol, which is followed by secondary changes in blood and tissues, such as hyperglycemia, hyperlactemia, hypercholesterolemia, changes in blood plasma enzyme activity and ion concentrations, reduced glycogen content in muscle and liver, an increase in metabolic rate, and also shifts in haematological profile and immunological capacity (Mazeaud et al. 1977; Staurnes et al. 1994; Stave and Robertson 1985).

Transporting live fish is a multiple-phase operation that should be designed to minimize stress and the costs involved. Transport of fish by tank trucks requires a special care to ensure that water quality parameters (temperature and oxygen content, in particular) and fish density requirements are maintained. Transport stress may be often caused by low oxygen levels, temperature differences between storage pond and tank water, or poor transport water quality due to inadequate water exchange that cause accumulation of carbon dioxide and ammonia (Erikson et al. 1997). Excretory products, mucus and regurgitated food degrade water quality. Respiration causes a decrease in dissolved oxygen levels and an increase in carbon dioxide levels in the transport medium. The excretion of nitrogenous wastes increases the level of ammonia in the transport medium. The increase in carbon dioxide concentration causes water pH to decrease.

Address for correspondence:
MVDr. Radka Dobšíková, Ph.D.
University of Veterinary and Pharmaceutical Sciences
Palackého 1-3, 612 42 Brno
Czech Republic

Phone: + 420 541 562 784
Fax: + 420 541 562 790
E-mail: dobsikovar@vfu.cz
http://www.vfu.cz/acta-vet/actavet.htm

Stress and muscle activity during transportation procedures usually shorten the time to the onset of rigor mortis, which is essentially triggered by the depletion of glycogen and ATP in muscle cells. Handling and processing of fish during rigor mortis can result in a loss of quality and lower fillet yield. The pre-rigor period must be long enough to ensure that bleeding, gutting, washing, chilling and packing take place before the onset of rigor mortis. Ante-mortem handling stress has also adverse effects on product quality such as reducing fish freshness and softening muscle texture (Izquierdo-Pulido et al. 1992; Nakayama et al. 1994).

The objective of this study was to assess the transport stress response, i.e. the effect of pre-transport manipulation procedures (hauling, netting, handling and loading) and transportation itself on changes in selected biochemical and haematological indices of three-year-old common carp (*Cyprinus carpio* L.) during a long-distance transportation. Transport water quality parameters were also monitored.

Materials and Methods

The experiment was carried out during a regular transport (April 29, 2005) of fish from storage ponds at a commercial fish farm in Hluboká nad Vltavou via Brno (Czech Republic) to a fish-pond in Boheľov (Slovakia). Carp were held in a freshwater flow-through storage pond for three days prior to transport, and then transferred to 2.4 m^3 transport tanks (loading density of 334 kg body weight·m^{-3}). Fish were transported in long-distance transporting tanks (AGK Kronawitter GmbH, Germany) that were insulated and continuously aerated with gas oxygen during the transport. The transport took 12 hours in total, 7 hours from Hluboká nad Vltavou to Brno and 5 hours from Brno to Boheľov.

Water quality variables were registered throughout the experiment, the results are given in Table 1. The values of temperature and oxygen content were measured *in situ* (WTW pH 235), and pH, NH_4^+, Cl^-, acid neutralisation capacity ($ANC_{4.5}$) and chemical oxygen demand (COD_{Mn}) were measured in laboratory.

The fish population tested consisted of 47 fish. Blood samples were withdrawn by cardiocenthesis before transport (i.e. during fish transfer from the storage pond to truck tanks) in Hluboká nad Vltavou (16 individuals), and after 7 and 12 hours of transport, in Brno (15 individuals) and Boheľov (16 individuals), respectively. *In situ*, haematocrit (PCV) was measured and leukogram blood smear prepared. A small volume of heparinized blood was reserved at 4 °C for red and white blood cell counts (RBC, WBC) and haemoglobin content (Hb) (Svobodová et al. 1991) . The rest of heparinized blood was centrifuged at 3000 rpm for 10 min and plasma samples were stored at 4 °C in Eppendorf test-tubes until analyses were performed (i.e. within 8 hours after blood sampling).

Plasma biochemical indices (glucose, lactate, LDH, CK, ALT, AST, ALP, ammonia, chloride, total protein) were measured by a biochemical analyzer Cobas EMira using commercial test kits. Plasma cortisol concentration was measured using HPLC/DAD (Waters).

Experimental data were statistically tested using variance analysis of the Statistica 6.0 software (Kruskal Wallis ANOVA).

Results

The results of water samples analysis are given in Table 1 and the values (mean ± SD) of biochemical and haematological indices of the common carp tested are presented in Table 2 and Table 3, respectively.

Table 1. Variables of the transport water tested (controlled aeration).

Parameters	Hluboká	Brno	Boheľov
temperature (ºC)	12.6	12.1	12.0
oxygen (%)	21.9	55.7	116.2
pH	7.39	6.46	6.57
$ANC_{4.5}$ (mmol·l^{-1})	2.0	2.0	2.2
COD_{Mn} (mg·l^{-1})	12.8	29.7	44.8
NH_4^+ (mg·l^{-1})	1.19	8.69	13.00
Cl^- (mg·l^{-1})	27.37	35.42	40.25

Mean values of body length, body weight and spleen weight (41.1 ± 4.13 cm, 1213.1 ± 322.07 g and 3.1 ± 0.93 g in Hluboká nad Vltavou, 39.6 ± 5.41 cm, 1203.3 ± 540.99 g and 4.1 ± 1.74 g in Brno, and 40.0 ± 3.89 cm, 1196.9 ± 367.18 g and 3.9 ± 1.32 in Boheľov, respectively) did not differ statistically among the groups tested.

In the study, the value of SSI ("spleen/soma index", i.e. relative weight of spleen) was also tested. The values of relative spleen weight were found 0.27 ± 0.088%, 0.34 ± 0.089%, and 0.33 ± 0.077% in Hluboká nad Vltavou, Brno, and Boheľov, respectively. No significant difference in the SSI indice among the groups tested was found.

Results of plasma biochemical profile of experimental groups of common carp exposed to long-distance transportation (Fig. 1) showed a significant increase in ammonia ($p < 0.01$), and a significant decrease in chloride ($p < 0.05$), lactate ($p < 0.05$), ALT ($p < 0.05$) and ALP ($p < 0.01$) values. The values of LDH ($p < 0.01$), AST ($p < 0.05$) and CK ($p < 0.01$) were significantly influenced by the transport, too, but no time-dependent relation was found. The concentrations of cortisol, glucose and total protein were not significantly changed (Table 2).

Table 2. Biochemical indices of common carp during long-distance transportation.

Indices	Hluboká Mean ± SD	Brno Mean ± SD	Boheľov Mean ± SD
Cortisol (ng·ml^{-1})	213.3 ± 61.88 ^{a}	206.6 ± 42.48 ^{a}	201.6 ± 36.38 ^{a}
Glucose (mmol·l^{-1})	8.2 ± 1.50 ^{a}	8.7 ± 2.20^{a}	9.4 ± 2.58 ^{a}
LDH (μkat·l^{-1})	10.2 ± 3.09 ^{a}	14.7 ± 0.89 ^{b}	9.9 ± 6.16 ^{a}
AST (μkat·l^{-1})	2.2 ± 1.17 ^{a}	3.4 ± 0.81 ^{b}	2.5 ± 1.23 ^{a}
CK (μkat.l^{-1})	375.1 ± 137.46 ^{a}	761.8 ± 457.44 ^{bc}	588.2 ± 411.57 ^{ac}
Total protein (g·l^{-1})	29.8 ± 3.43 ^{a}	31.1 ± 5.46 ^{a}	30.1 ± 3.46 ^{a}

Note: Groups with different alphabetic superscripts differ significantly.

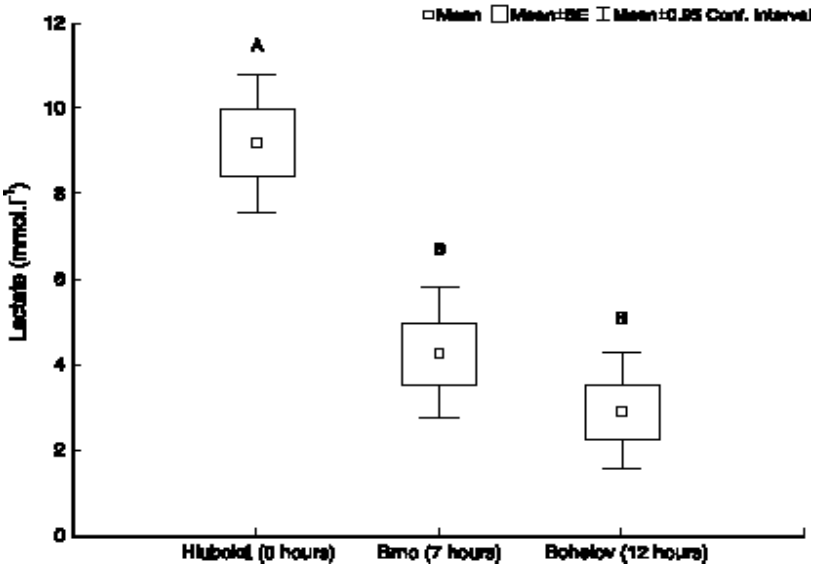

Fig. 1a. Plasma lactate concentration of common carp during long-distance transportation.
Note: Columns with different alphabetic superscripts differ significantly.

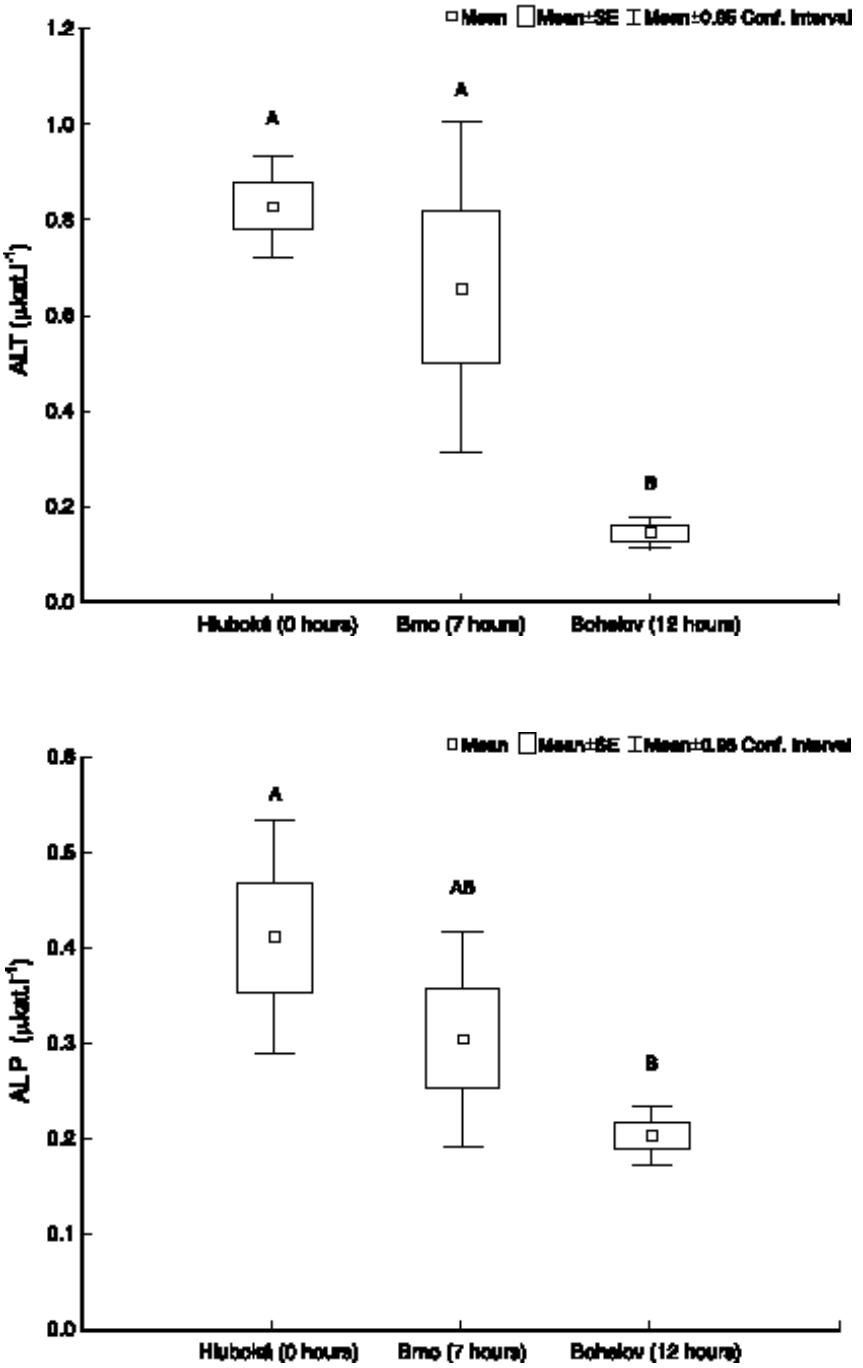

Fig. 1b, c. Plasma ALT and ALP activities of common carp during long-distance transportation.
Note: Columns with different alphabetic superscripts differ significantly.

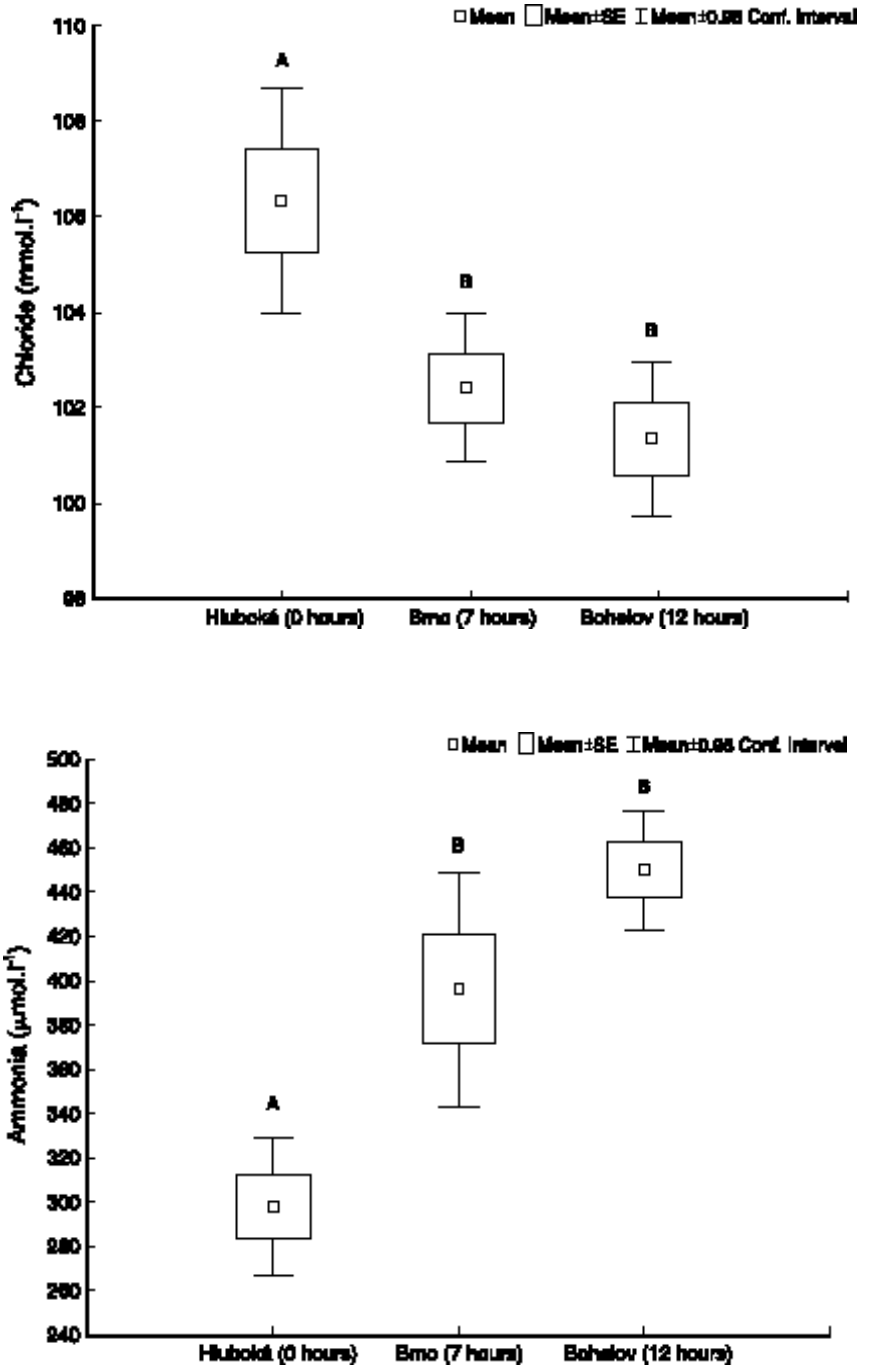

Fig. 1 d, e. Plasma chloride and ammonia concentrations of common carp during long-distance transportation.
Note: Columns with different alphabetic superscripts differ significantly.

As far as the haematological indices are concerned (Fig. 2), the long-distance transport caused a significant increase in MCV ($p < 0.01$), metamyelocytes counts ($p < 0.05$) and band neutrophils counts ($p < 0.01$). The values of PCV ($p < 0.05$) were also significantly changed without the confirmation of time-dependent relation. The other haematological indices tested were comparable within the groups (Table 3).

Table 3 Haematological indices of common carp during long- distance transportation.

Indices	Hluboká Mean ± SD	Brno Mean ± SD	Bohelov Mean ± SD
RBC ($T \cdot l^{-1}$)	1.46 ± 0.175 [a]	1.46 ± 0.193 [a]	1.38 ± 0.225 [a]
PCV ($l \cdot l^{-1}$)	0.32 ± 0.027 [a]	0.36 ± 0.044 [b]	0.36 ± 0.028 [b]
Hb ($g \cdot l^{-1}$)	94.74 ± 28.047 [a]	92.68 ± 15.463 [a]	93.43 ± 9.875 [a]
MCH (pg)	65.49 ± 21.108 [a]	64.02 ± 9.772 [a]	69.11 ± 12.329 [a]
MCHC ($l \cdot l^{-1}$)	0.30 ± 0.084 [a]	0.27 ± 0.048 [a]	0.26 ± 0.028 [a]
WBC ($G \cdot l^{-1}$)	57.56 ± 32.932 [a]	76.33 ± 45.613 [a]	79.94 ± 28.963 [a]
Lymphocytes ($G \cdot l^{-1}$)	51.02 ± 33.147 [a]	65.32 ± 42.681 [a]	63.74 ± 26.855 [a]
Monocytes ($G \cdot l^{-1}$)	0.91 ± 0.678 [a]	0.73 ± 0.968 [a]	0.52 ± 1.494 [a]
Myelocytes ($G \cdot l^{-1}$)	1.98 ± 1.944 [a]	2.17 ± 1.500 [a]	2.74 ± 1.995 [a]
Segmented neutrophils ($G \cdot l^{-1}$)	0.82 ± 1.047 [a]	0.92 ± 1.003 [a]	0.62 ± 0.988 [a]
Basophils ($G \cdot l^{-1}$)	0.03 ± 0.089 [a]	0.00 ± 0.000 [a]	0.13 ± 0.392 [a]

Note: Groups with different alphabetic superscripts differ significantly.

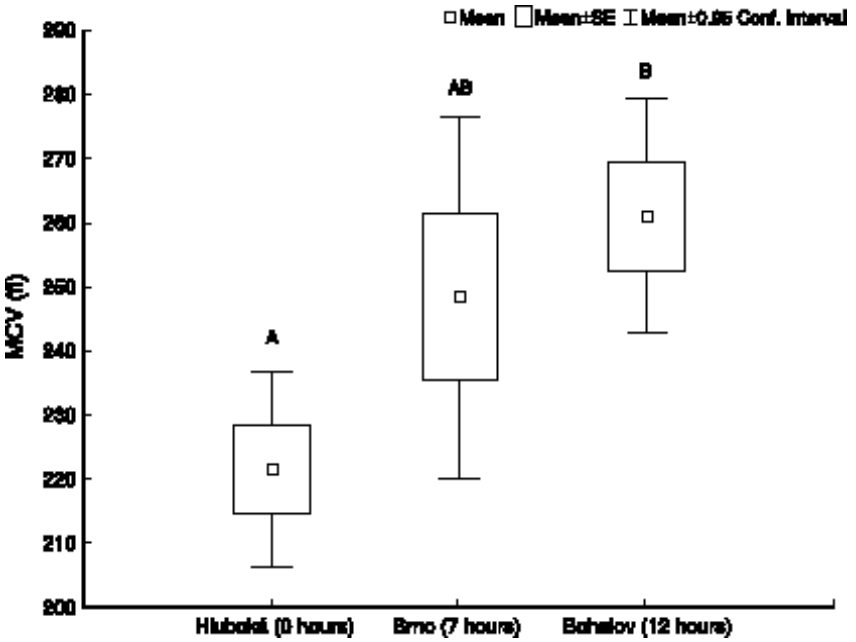

Fig. 2a. MCV value of common carp during long-distance transportation.
Note: Columns with different alphabetic superscripts differ significantly.

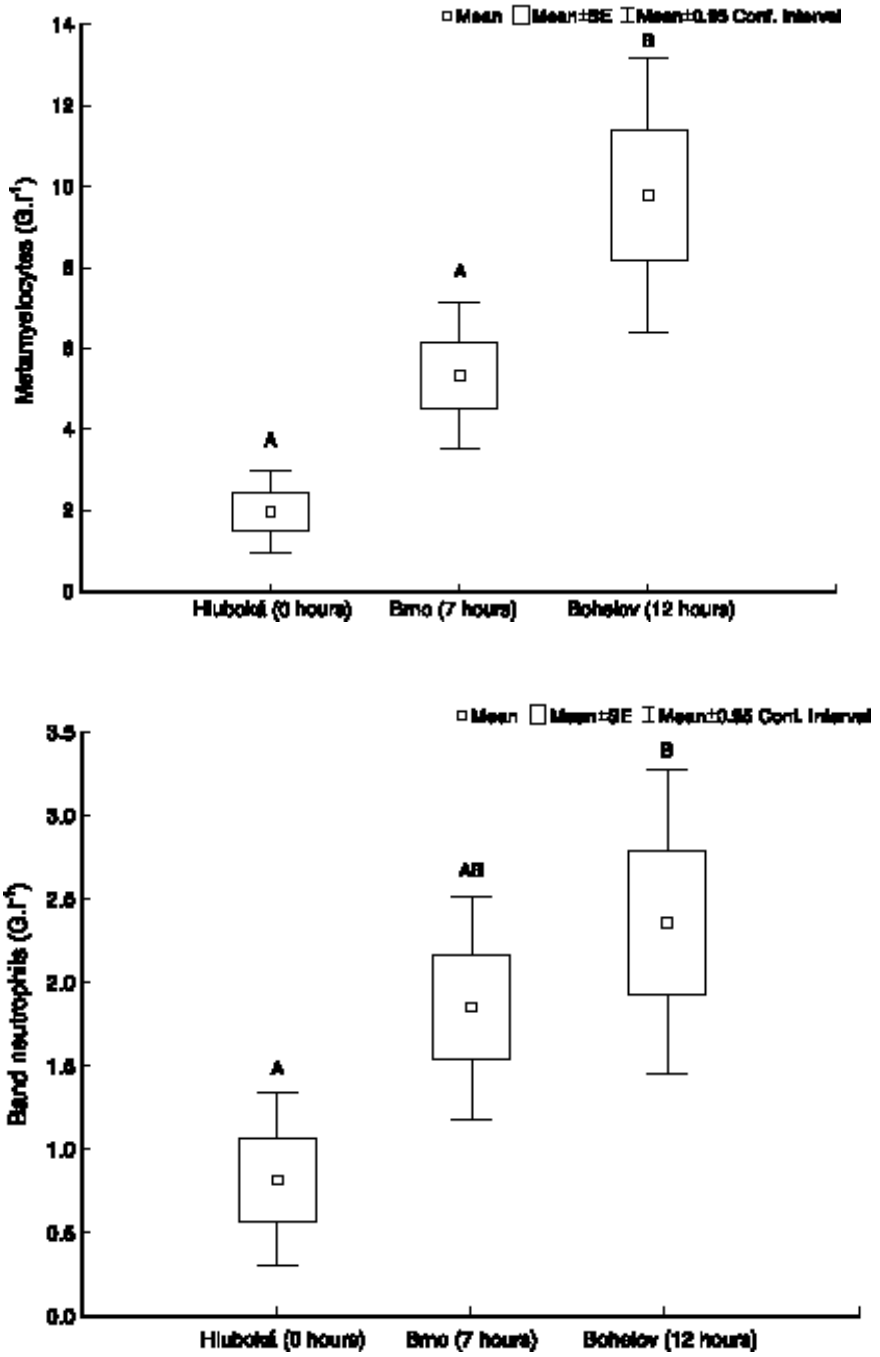

Fig. 2b, c. Metamyelocytes counts, and band neutrophil counts of common carp during long-distance transportation.
Note: Columns with different alphabetic superscripts differ significantly.

Discussion

Recent studies show that one of the most important factors that affect the fish welfare during short- or a long-distance transport, is an adequate adjustment and stress reduction in fish by minimization of manipulation procedures, pre-transport defecation to prevent the accumulation of metabolites in transport medium, as well as the avoidance of temperature and light changes, using anaesthetics, etc. (Svobodová et al. 1999) .

None of the water-quality parameters measured (Table 1) suggested that adverse water quality prevailed at any time during long-distance transportation.

Being vulnerable to short-term temperature fluctuations, necessary care must be taken when transferring fish to new ponds or new culture or transport media until acclimatized (Roberts and Shepherd 1997). In our experiment, storage pond and truck tank water temperatures were found similar (i.e. approximately 12.5 °C). As for common carp, a critical value of oxygen saturation in water is 12.0% and 15.3% at 10 and 15 °C, respectively (Svobodová et al. 2003). In the study, transport water oxygen content increased markedly during the fish transportation due to continual aeration, and was sufficient for the fish.

The value of pH slightly decreased during the transport, nevertheless, it held within the optimum range 6.4 - 8.4 (Roberts and Shepherd 1997). The elevated COD_{Mn} value and the levels of ammonia and chloride in transport medium may be ascribed to higher fish metabolic rate in a relatively high-density tank volume.

Pre-transport manipulation, i.e. capturing and reloading fish into transport tanks, appeared to be a severe stressor to fish. In accordance with our findings, some previous studies proved that capture-loading manipulation (hauling, netting, catching, reloading) is the major cause of the transport stress response (Schreck et al. 1989; Weireich and Tomasso 1991). Robertson et al. (1987), Pottinger (1998) and Ruane et al. (2001) reported that manipulation with fish in general (capturing, confinement, increased stocking density, transportation, etc.) lead to the elevation of plasma cortisol, glucose and lactate concentrations. Ruane et al. (2001) reported that during net-confinement, plasma cortisol levels increased rapidly after 30 min to reach the concentrations of 240 - 320 $ng \cdot ml^{-1}$. Robertson et al. (1987) reported that cortisol concentrations increased with the increase of transport time. On the basis of our study results, we assume that pre-transport manipulation lead to the increase of plasma cortisol. The increased concentration of cortisol lasted throughout the transportation.

Plasma glucose levels are elevated in stressed fish as a consequence of the increase of blood catecholamine levels. Nevertheless, the index is a more equivocal index of stress than cortisol, as plasma glucose is a function of many factors (diet, age, season, etc.). In our study, glucose levels were found very high at the beginning, and slightly (non-significantly) increased during the test. The increase of glucose level at the beginning of the test may be ascribed to an enhanced stress reaction of carp to reloading processes, when fish were kept in nets in a high density. Glucose level in unstressed carp reported in previous studies ranged within 2.8 - 5.6 $mmol \cdot l^{-1}$ (Hertz et al. 1989), 2.0 - 5.0 $mmol \cdot l^{-1}$ (Svobodová et al. 1991) and 2.5 - 3.6 $mmol \cdot l^{-1}$ (Blasko et al. 1992). Carp exposure to prolonged hypoxia, crowding stress and confinement in anglers' keepnets were reported to increase blood glucose levels from 5.0 to 10.0 $mmol \cdot l^{-1}$ (van Raaij et al. 1996), from 4.7 to 6.9 $mmol \cdot l^{-1}$ (Yin et al. 1995), and from 5.1 $mmol \cdot l^{-1}$ (3.6 and 3.3 $mmol \cdot l^{-1}$, respectively) to 8.0 $mmol \cdot l^{-1}$ (7.3 and 6.8 $mmol \cdot l^{-1}$, respectively) in three experiments of Pottinger (1998).

The elevation of plasma lactate levels follows on respiratory activity under anaerobic conditions and reflects the imposition of severe exercise (Pottinger 1998). In our study, the highest level of plasma lactate (9.17 $mmol \cdot l^{-1}$) was found at the beginning of the test, when the fish were stressed by hauling and exhausted by physical exercise in relatively hypoxic conditions. In Pottinger's study (1998), the confinement of fish in keepnets

caused significant elevation of plasma lactate from 3.0 to 13.9 mmol·l^{-1} and from 2.0 to 3.7 mmol·l^{-1}. In our study, lactate levels decreased significantly ($p < 0.05$) to baseline levels (2.89 mmol·l^{-1}) during the transport, that indicates that the fish became calm after reloading procedure. The decrease of plasma lactate concentration during transportation also corresponds to the increase of water oxygen content in truck tanks.

At the end of the transport, a significant decrease in ALT ($p < 0.05$), ALP ($p < 0.01$) and chloride ($p < 0.05$) levels, and a significant increase in ammonia ($p < 0.01$) concentration were found. Initial high ALT, ALP and chloride levels may be ascribed to the enhancement of fish basal metabolism during stressful reloading. Subsequent modulation of fish during transport leads to a decrease in their levels. Davison et al. (1994) reported that hypoxic conditions caused an increase in chloride levels in fish. That corresponds to the results of our study, in which the highest chloride concentration was found in fish during hauling procedure, when the lowest water oxygen content was measured. During the transport, the oxygen content increased rapidly and chloride levels dropped significantly.

During exhaustive exercise (attack of prey, escape from predators, locomotion and migration processed, starvation), an increase of active energy demand occurs in fish (Smutná et al. 2002). Direct deamination of important tissue energy sources, i.e. amino acids, especially glutamate and aspartate (liver, muscle), histidine (kidney, muscle), serine (kidney) and glutamine (liver, kidney, muscle), leads to energy production (catabolism of glutamine and amino acids provides ATP molecules), and simultaneously to an increase of the ammonia level in fish (Smutná et al. 2002; Philip and Rajasree 1996). Production of an active actine-myosine complex, necessary for muscle contraction, leads to the splitting of ATP to ADP and P_i. Deamination of the AMP molecule, produced from the ADP molecule, leads to the production of IMP and ammonia. It means that in fish, burst exercise (i.e. high muscle activity) results in the production of ammonia. When the detoxification capabilities of the fish are exceeded, ammonia cumulates in the fish organism and acts as a toxicant (Smutná et al. 2002). In our study, the increase of ammonia level was caused by the enhancement of basic energy demand of fish transported (the increase of deamination processes in tissues), and by higher muscle activities of fish in space-quartered transport tanks.

Changes in haematological profile of peripheral blood have often been used as an indicator of stress exposure, though haematological indices results reported are found equivocal. Serious changes in haemogram are found in fish exposed to acute or chronic stress (Svobodová et al. 1994). As far as haematological indices of our study are concerned, twelve-hour transportation caused a significant increase in MCV level ($p<0.01$), metamyelocytes ($p < 0.05$) and band neutrophils ($p < 0.01$) .

In the blood of stressed fish, an increase in RBC, haemoglobin concentration and haematocrit level are observed (Doubek et al. 2003). Haemoglobin and haematocrit are often elevated during stress situations to increase oxygen carrying capacity and oxygen supply to the major organs in response to higher metabolic demands (Ruane et al. 1999). In our study, the highest RBC value was found in fish blood samples withdrawn during the transfer from storage ponds to transport tanks in Hluboká, when stress-induced RBC release from spleen to blood circulation was reported. RBC values then slightly decreased. Hb level was not affected, haematocrit and MCV values increased significantly.

Ruane et al. (2001) tested the effect of three-hour net confinement on common carp and found no specific changes in haemoglobin content. Haematocrit values decreased non-significantly after 0.5h and 1.0h confinement. During the recovery, the haematocrit levels increased and became comparable to control levels. The lowest haematocrit values of carp tested in our study were found during reloading fish into transport tanks. Haematocrit values increased significantly during the transportation.

Ruane et al. (2002) exposed common carp to high stocking densities of 56.8 and 113.6 $kg \cdot m^{-3}$ for a period of 87 hours without finding any significant high-density effect on fish haematocrit and haemoglobin values. No changes in haematocrit values were also recorded in the Atlantic cod transport experiment by Staurnes et al. (1994).

Physiological responses of matrinxa juveniles (*Brycon cephalus*) juveniles were determined after the procedures of capturing, loading and 4h transport at different densities (Urbinati et al. 2004). Haematocrit increased after loading, maintaining levels slightly high until the end of the experiment. No differences were verified in RBC number. Acerete et al. (2004) found an increase in haematocrit values and RBC after acute handling stress.

Results of white blood line observation showed a significant increase in counts of metamyelocytes ($p < 0.05$) and band neutrophils ($p < 0.01$), as well as a slight increase in leukocytes and myelocytes, and monocytes count reduction (both non-significant).

An important secondary effect of stress reported in fish (as a consequence of stress-related release of catecholamines) is immunosuppression (lymphocytopenia) and neutrophilia (Wiik et al. 1989; Svoboda 2001; Engelsma et al. 2003). Transport- and handling-stress leads to the elevation of plasma cortisol that is reported to have a direct cytolytic effect on lymphocytes (Wiik et al. 1989). These findings are in agreement with the data published by Wendelaar (1997), who reported that stress caused a rapid increase in neutrophils and a reduction of lymphocytes in peripheral blood. Reduction in lymphocyte count in stressed fish may partly be due to the extravasation of the cells and their penetration to the epithelium of gills, skin or intestine.

In our experiment, long-distance transport in high density tanks led to a slight increase of leukocyte count in the fish tested. The stress was also expressed in the increase in neutrophil granulocytes, mainly of juvenile forms (metamyelocytes and band neutrophils). Ortuno et al. (2001) reported that intense short-term crowding stress caused leukocytes release from head-kidney and their cumulation in blood circulation. Pulsford et al. (1994) detected an increased number of leukocytes, particularly phagocytes and damaged cells, in peripheral blood of stressed dab, *Limanda limanda*, along with a decrease in lymphocyte, thrombocyte and erythrocyte counts. In Espelid et al. (1996) study, after cortisol administration, a marked increase in the relative number of thrombocytes was reported, whereas granulocyte, monocyte and lymphocyte counts remained still relatively constant in Atlantic salmon.

In conclusion, pre-transport manipulation procedures were found to be very stress-inducing and transport itself was relatively considerate for common carp. Although carp may quickly adapt to ambient environment, disturbances should be kept to a minimum to ensure optimal transport conditions, and subsequently high quality standards of fish products.

Stresové zatížení kapra obecného (*Cyprinus carpio* L.) při dlouhodobém transportu

U tříletého kapra obecného byla sledována odpověď, tj. změny vybraných biochemických a hematologických parametrů, na stresové zatížení při dlouhodobém transportu ve speciálních transportních tancích. Dvanáctihodinový transport vedl k signifikantnímu zvýšení koncentrace amoniaku ($p < 0.01$), hodnoty MCV ($p < 0.01$), počtu metamyelocytů ($p < 0.05$) a neutrofilů - tyček ($p < 0.01$) a k signifikantnímu poklesu koncentrace Cl^- ($p < 0.05$) a laktátu ($p < 0.05$) a katalytické koncentrace ALT ($p < 0.05$) a ALP ($p < 0.01$). Katalytická koncentrace LDH ($p < 0.01$), AST ($p < 0.05$) a CK ($p < 0.01$) a hodnota PCV ($p < 0.05$) byly transportem statisticky významně ovlivněny, avšak nebyla prokázána časová závislost na době transportu. Hladiny kortisolu, glukosy a celkových proteinů v biochemickém profilu a hodnoty Hb, MCH, MCHC a počet erytrocytů, leukocytů a leukogram (vyjma metamyelocytů a neutrofilů - tyček) v hematologickém profilu ryb nebyly

transportem signifikantně ovlivněny. Manipulační procesy spojené s nakládkou ryb byly pro testované ryby stresujícím faktorem, vlastní transport byl relativně šetrný.

Acknowledgements

This research was supported by the Ministry of Education, Youth and Sports of the Czech Republic (MSM project No. 6215712402).

References

ACERETE L, BALASCH JC, ESPINOSA E, JOSA A, TORT L 2004: Physiological responses in Eurasian perch (*Perca fluviatilis*, L.) subjected to stress by transport and handling. Aquaculture **237**: 167-178

BARTON BA, PETER RE, PAULENCU CR 1980: Plasma cortisol levels of fingerling rainbow trout (*Salmo gairdneri*) at rest, and subjected to handling confinement, transport and stocking. Can J Fish Aquat Sci **37**: 805-811

BLASKO J, FERNANDEZ J, GUTIERREZ J 1992: Variations in tissue reserves, plasma metabolites and pancreatic hormones during fasting in immature carp (*Cyprinus carpio*). Comp Biochem Physiol A **103**: 357-363

DAVISON W, FRANKLIN CE, MC KENZIE JC 1994: Haematological changes in an Antarctic teleost, *Trematomus bernacchii*, following stress. Polar Biol **14**: 463-466

DOUBEK J, BOUDA J, DOUBEK M, FÜRLL M, KNOTKOVÁ Z, PEJŘILOVÁ S, PRAVDA D, SCHEER P, SVOBODOVÁ Z, VODIČKA R 2003: Veterinární hematologie. Noviko Brno, 464 p. (in Czech).

ENGELSMA MY, HOUGEE S, NAP D, HOFENK M, ROMBOUT JH, VAN MUISWINKEL WB, LIDY VERBURG-VAN KEMENADE BM 2003: Multiple acute temperature stress affects leukocyte populations and antibody response in common carp, *Cyprinus carpio* L. Fish Shellfish Immun **15**: 397-410

ERIKSON U, SIGHOLT T, SELAND A 1997: Handling stress and water quality during live transportation and slaughter of Atlantic salmon (*Salmo salar*). Aquaculture **149**: 243-252

ESPELID S, L∈KKEN GB, STEIRO K, BØGWALD 1996: Effect of cortisol and stress on the immune system in Atlantic salmon (*Salmo salar* L.). Fish Shellfish Immun **6**: 95-110

HERTZ Y, MADAR Z, HEPHER B, BERTLER A 1989: Glucose metabolism in the common carp (*Cyprinus carpio* L.): the effects of cobalt and chromium. Aquaculture **76**: 255-267

IZQUIERDO-PULIDO ML, HATAE K, HAARD NF 1992: Nucleotide catabolism and changes in texture indices during ice storage of cultured sturgeon, *Acipenser transmontanus*. J Food Biochem **16**: 173-192

MAZEAUD MM, MAZEAUD F, DONALDSON EM 1977: Primary and secondary effects of stress in fish: some new data with a general review. Amer Fish Soc **106**: 201-212

NAKAYAMA T, TOYODA T, OOI A 1994: Physical property of carp muscle during rigor tension generation. Fisheries Sci **60**: 717-721

ORTUNO J, ESTEBAN MA, MESEGUER J 2001: Effects of short-term crowding stress on the gilthead seabream (*Sparus aurata* L.) innate immune response. Fish Shellfish Immun **11**: 187-197

PHILIP GH, RAJASREE BH 1996: Action of cypermethrin on tissue transamination during nitrogen metabolism in *Cyprinus carpio*. Ecotox Environ Safe **34**:174-179

POTTINGER TG 1998: Changes in blood cortisol, glucose and lactate in carp retained in anglers' keepnets. J Fish Biol **53**: 728-742

PULSFORD AL, LEMAIRE-GONY S, TOMLINSON M, COLLINGWOOD N, GLYNN PJ 1994: Effects of acute stress on the immune system of the dab, *Limanda limanda*. Comp Biochem Physiol C **109**: 129-139

ROBERTS RJ, SHEPHERD CJ 1997: Handbook of Trout and Salmon Disease. 3rd Edition. Blackwell Science Ltd., Oxford, 179 p.

ROBERTSON L, THOMAS P, ARNOLD CR 1987: Plasma cortisol and secondary stress response of cultured red drum (*Sciaenops ocellatus*) to several transportation procedures. Aquaculture **68** : 115-130

RUANE MN, CARBALLO EC, KOMEN J 2002: Increased stocking density influences the acute physiological stress response of common carp *Cyprinus carpio* (L.). Aquac Res **33**: 777-784

RUANE NM, HUISMAN EA, KOMEN J 2001: Plasma cortisol and metabolite level profiles in two isogenic strains of common carp during confinement. J Fish Biol **59**: 1-12

RUANE NM, WENDELAAR BONGA SE, BALM PHM 1999: Differences between rainbow trout and brown trout in the regulation of the pituitary-interrenal axis and physiological performance during confinement. Gen Comp Endoc **115**: 210-219

SCHRECK CB, SOLAZZI MF, JOHNSON SL, NICKELSON TE 1989: Transportation stress affects performance of coho salmon, *Oncorhynchus kisutch*. Aquaculture **82**: 15-20

SMUTNÁ M, VORLOVÁ L, SVOBODOVÁ Z 2002: Pathobiochemistry of ammonia in the internal environment of fish (Review). Acta Vet Brno **71**: 169-181

STAVE JW, ROBERTSON BS 1985: Hydrocortisone suppresses the chemiluminescent response of striped bass phagocytes. Dev Comp Immunol **9**: 77-84

SVOBODA M 2001: Stress in fish - a review. Bull RIFCH Vodňany **37**: 169-191 (in Czech)

SVOBODOVÁ Z, KALÁB B, DUŠEK L, VYKUSOVÁ B, KOLÁŘOVÁ J, JANOUŠKOVÁ D 1999. The effect of handling and transport on the concentration of glucose and cortisol in blood plasma of common carp. Acta Vet Brno **68**: 265-274

SVOBODOVÁ Z, MÁCHOVÁ J, VESELÝ V, MODRÁ H, SVOBODA M 2003: Veterinary toxicology. Laboratories. Part I. UVPS Brno, 179 p. (in Czech).

SVOBODOVÁ Z, PRAVDA D, PALÁČKOVÁ J 1991: Unified methods of haematological examination of fish. Methods No. 20. Research Institute of Fish Culture and Hydrobiology, Vodňany, 31 p.

SVOBODOVÁ Z, VYKUSOVÁ B, MÁCHOVÁ J 1994: The effect of pollutants on selected haematological and biochemical parameters in fish. In: MÜLLER R, LLOYD R (Eds.): Sublethal and chronic effects of pollutants on freshwater fish. FAO - Fishing News Books, Cambridge, pp. 39-52.

URBINATI EC, DE ABREU JS, DA SILVA CAMARGO AC, PARRA MAL 2004: Loading and transport stress of juvenile matrinxa (*Brycon cephalus*, Characidae) at various densities. Aquaculture **229**: 389-400

VAN RAAIJ MTM, VAN DEN THILLARTM GEEJ, VIANEN GJ, PIT DSS, BALM PHM, STEFFENS AB 1996: Substrate mobilization and hormone changes in rainbow trout (*Oncorhynchus mykiss*, L.) and common carp (*Cyprinus carpio*, L.) during deep hypoxia and subsequent recovery. J Comp Physiol B **166**: 443-452

WEIREICH CR, TOMASSO JR 1991: Confinement- and transport- induced stress on red drum juveniles: effect of salinity. Prog Fish Cult **53**: 146-149

WENDELAAR SE 1997: The stress response in fish. Physiol Rev **77**: 591-625

WIIK E, ANDERSEN K, UGLENES I, EGIDIUS E 1989: Cortisol-induced increase in susceptibility of Atlantic salmon, *Salmo salar*, to *Vibrio salmonicida*, together with the effects on the blood cell paterns. Aquaculture **83**: 201-215

YIN Z, LAM TJ, SIN YM 1995: The effect of crowding stress on the non-specific immune response in fancy carp (*Cyprinus carpio* L.). Fish Shellfish Immun **5**: 519-528

ACTA VET. BRNO 2006, 75: 449-454; doi:10.2754/avb200675030449

Mortality in Dairy Cows Transported to Slaughter as Affected by Travel Distance and Seasonality

V. VEČEREK[1], M. MALENA jr.[2], M. MALENA[2], E. VOSLÁŘOVÁ[1], I. BEDÁŇOVÁ[1]

[1] University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic
[2] State Veterinary Administration, Czech Republic

Received November 16, 2005
Accepted May 11, 2006

Abstract

Večerek V., M. Malena jr., M. Malena, E. Voslářová, I. Bedáňová: Mortality in Dairy Cows Transported to Slaughter as Affected by Travel Distance and Seasonality. Acta Vet. Brno 2006, 75: 449-454.

A significant lapse in welfare conditions of dairy cows being shipped to slaughter may result in higher death rates of the animals in the course of transportation.

The objective of this work was to quantify the rate at which dairy cows die while transported to slaughter facilities, to determine the impact of travel distance on this rate, the seasonal effect on the number of deceased cows, and to identify any developmental trends associated with these losses.

In the period of 1997 - 2004, veterinary inspectors in the Czech Republic tracked the deaths of dairy cows in connection with their transportation to slaughter. Of the total number of 1,107,685 monitored dairy cows transported to slaughter in the Czech Republic in the period from 1997 to 2004, 418 dairy cows died. The death rate was determined to be 0.038%. However, the mortality varied depending on the distance travelled, from 0.013% for trips not exceeding 50 km, to 0.183% for trips longer than 300 km.

Comparing Period I (1997 - 2000) with Period II (2001 - 2004), the overall death rate of dairy cows attributable to slaughterhouse-bound transportation rose from 0.023% to 0.057% (a 2.51 index) and the difference between periods was highly significant ($p < 0.01$). This outcome revealed an undesirable long-range trend of rising dairy cow mortality in all travel distances.

The obtained results document a relatively low sensitivity of dairy cows to stresses arising from shipment to slaughter as indicated by transportation-related deaths. However, the growing trend in the number of dairy cows dying on their way to slaughter is a warning sign in relation to the welfare of cows subjected to such transportation.

Cattle, death rate, welfare, stress, journey time, summer months, winter months

Poor welfare of dairy cows during transport is accompanied by a significant rise of stress levels in the animals prior to slaughter, and may be reflected in a higher death rate of the cows undergoing transportation. The number of animals dying en route and shortly after delivery to the abattoir may serve as an indicator that the transport experienced a grave disruption of welfare conditions.

Cattle welfare during its transportation to a slaughterhouse in terms of stress levels in the animals was studied by von Holleben et al. (2003). They monitored the handling of slaughter-bound cattle as it travelled in Germany.

A stress load in cows manifests itself by changes in blood-based indicators of stress. Mudroň et al. (2005) studied stress response in dairy cows as related to glucose in blood. The secretion of adrenocorticotrophin from peripheral bovine lymphocytes under transport-induced stress was studied by Dixit et al. (2001). The influence of transport stress on peripheral blood neutrophils and on somatic cell counts in milk was analyzed by Yagi et al. (2004). Verkerk et al. (1998) studied the influence of transport stress on milk cortisol concentrations in lactating dairy cows. Morrow et al. (2002) were concerned with the effects of transport-related stress on faecal glucocorticoid metabolites in dairy cattle. Likewise, Palme et al. (2000) and Most et al. (2002) considered the effects of transport-related stress on faecal cortisol metabolite concentrations.

Address for correspondence:
Prof. MVDr. Vladimír Večerek, CSc.
University of Veterinary and Pharmaceutical Sciences Brno
Palackého 1-3
612 42 Brno, Czech Republic

Phone: +420 541 562 770
Fax: +420 541 562 790
E-mail:vecerek@vfu.cz
http://www.vfu.cz/acta-vet/actavet.htm

Broom (2003) scrutinized transport-generated stresses in cattle in detail, inclusive of physiological, ethological and other indicators.

Transport conditions affect the well-being of transported cattle. They also influence the occurrence of disorders of the locomotor apparatus in cattle due to transportation, the frequency of physical damage to the slaughter-bound animals, and the potential for lower carcass and meat quality. The occurrence of damage to the locomotor apparatus in cattle in consequence of improper handling during the transfer to slaughter was noted by Pištěková et al. (2004). A mechanical damage to the animals in the course of transportation, and the spread of processes triggered by cattle exposure to stress may be instrumental in veterinary decisions concerning the suitability of the meat for human consumption, and may increase the incidence of meat being considered unfit for that purpose because of a decline in sensory qualities, as reported by Večerek et al. (2003). The effect of distance involved in cattle transportation to a slaughter facility on carcass bruises was studied by Hoffman et al. (1998). Villarroel et al. (2003) were concerned about the effects of commercial transport on cattle welfare and meat quality in Spain.

Grandin (1999) concentrated on mitigating the stress levels in cattle by practicing low-stress cattle-handling techniques. Wikner et al. (2003) analyzed the factors affecting stress levels in transported cattle. In terms of behavioural analysis, they emphasised driving performance, proper handling during loading and unloading, and air quality inside the vehicle. Villarroel et al. (2003) considered the influence of various climatic conditions on the stress in cattle undergoing transportation. Among the stress factors, von Holleben et al. (2003) included also re-grouping, loading, transport time, loading density, mounting prevention, unloading, and lairage time.

The objective of this work was to establish the death rates prevalent in dairy cows shipped to slaughter facilities in the Czech Republic in the period of 1997 - 2004, to determine the effect of travel distance and season on these death rates, and, in addition, to identify any developmental trends in the numbers of dairy cows dying in the process of being transported to slaughter.

Materials and Methods

In the period of 1997 - 2004, State Veterinary Administration of the Czech Republic monitored the numbers of dairy cows shipped to facilities for regular slaughter. A record was kept of the cows deceased in connection with the transportation, namely the number of cows that expired directly in the conveyance or after delivery into the facility. The shipments of dairy cows intended for forced slaughter were not monitored, as the situation typically involved dairy cows in poor health, where the cow's possible death could be the consequence of an existing illness rather than its transfer to a slaughter facility.

A ratio of the dead animal count to the transported animal total was computed and expressed as a percentage for the entire period under observation, thus representing the overall dairy cow mortality associated with transportation to slaughter.

Also computed as percentages were ratios of the dead animal counts to the transported animal totals for travel distances of up to 50 km, 51 to 100 km, 101 to 200 km, 201 to 300 km and over 300 km. These results served to ascertain the effect of travel distance on the dairy cow mortality attributed to slaughterhouse transportation.

A ratio computed from the number of dairy cows that died and the transported total was stated as a percentage for the colder periods (the months of November, December, January, February, March, April) and for the warmer periods (the months of May, June, July, August, September, October). The results made it possible to quantify the seasonal effect on the mortality of dairy cows under transportation to slaughter facilities.

Relative numbers of the dairy cows that perished, expressed as percentages, were likewise computed for Period I (1997 - 2000) and Period II (2001 - 2004). An index representing a ratio of the deceased cow percentages in Period II versus Period I was also computed. The index value greater than 1.00 indicates a growing trend in the dairy cow mortality within the given period, the value of 1.00 indicates a trend where the dairy cow mortality neither rises nor falls within the given period, and the value lower than 1.00 indicates a decreasing trend in the dairy cow mortality within the given period.

Results of absolute and relative frequencies were processed using the statistical calculations module of the Excel software package. For statistical processing of results (χ^2 - test), Unistat 5.1. software was used.

Results

Table 1 shows the number of dairy cows deceased in the course of transportation to slaughter, arranged by travel distance to the slaughter facility. Table 1 shows that the overall rate at which dairy cows died in the process of being shipped to slaughter was 0.038% for the entire period of time under observation. The effect of travel distance on the mortality of dairy cows transported to slaughter became apparent from the fact that travel distances of up to 50 km had the death rate of 0.013%, whereas the long distances of over 300 km exhibited substantially higher rates of up to 0.183%. Clearly, the mortality of dairy cows increases with the distance travelled.

Table 1. Number of dairy cow deaths related to slaughterhouse transportation as a function of distance travelled

	Entire Monitored Period 1997 – 2004		
Distance (km)	Transported (number)	Deceased (number)	Mortality (%)
< 50	713383	95	0.013
51 - 100	231251	92	0.040
101 - 200	120100	165	0.137
201 - 300	33102	48	0.145
> 300	9849	18	0.183
Total	1107685	418	0.038

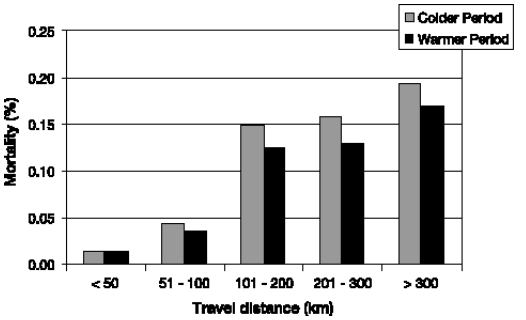

Fig. 1. Number of dairy cow deaths related to slaughterhouse transportation, by colder and warmer season

Fig. 1 shows the number of dairy cows deceased in the course of transportation to slaughter in colder and warmer seasons, listed by individual distance categories. Fig. 1 indicates that season is a factor in the death rates of dairy cows under transportation. Overall, the highest mortality occurred in the colder periods (the months of January, February, March, April, November, December), as opposed to the warmer periods (the months of May, June, July, August, September, October). Therefore, in terms of dairy cows dying in connection with transfers to slaughter facilities, the colder period has a greater negative influence on animal welfare than the warmer period, even if the difference between death rates of transported dairy cows in colder and warmer periods was not significant.

Table 2 shows the trends in the death rates of dairy cows transported to slaughter facilities, in correlation to travel distances. Table 2 shows that the time-related mortality trends, taken as an overall value and individually for different travel distances, are expressed by indices

higher than 1.00. Comparing the 1997 - 2000 period with the 2001 - 2004 period, the mortality rose in all distance categories and the difference was significant (except for travel distances over 300 km). This trend may be characterized as negative.

Table 2. Trends in dairy cow mortality related to slaughterhouse transportation as a function of travel distance

Travel distance (km)	Period I 1997 – 2000			Period II 2001 – 2004				*p*
			B			C	C/B	
	T (number)	D (number)	M (%)	T (number)	D (number)	M (%)	Trend (index)	
< 50	440132	46	0.010	273251	49	0.018	1.72	0.008**
51 - 100	117513	29	0.025	113738	63	0.055	2.24	0.000**
101 - 200	45686	43	0.094	74414	122	0.164	1.74	0.002**
201 - 300	15612	14	0.090	17490	34	0.194	2.17	0.012*
> 300	5731	10	0.174	4118	8	0.194	1.11	0.821
Total	624674	142	0.023	483011	276	0.057	2.51	0.000**

Legend:
T = number of transported dairy cows, D = number of deceased dairy cows during transport, M = mortality, *p* = statistical significance, * = significant ($p < 0.05$), ** = highly significant ($p < 0.01$)

Discussion

Shipment of dairy cows to slaughter imposes a significant stress on the transported animals. According to von Holleben et al. (2003), Mudroň et al. (2005), the stress manifests itself particularly by changes in behaviour, heart rate, and further by changes in the levels of cortisol, glucose, creatin kinase and other indicators. Dixit et al. (2001) mention changes in the secretion of adrenocorticotropin (ACTH) from peripheral bovine lymphocytes. Yagi et al. (2004) registered an increase in the migration capacity of peripheral blood neutrophils in dairy cows as a consequence of transport-related stress. Transport stress increases milk cortisol concentrations (Verkerk et al. 1998), somatic cell counts in milk (Yagi et al. 2004), and faecal cortisol metabolite concentrations (Palme et al. 2000; Morrow et al. 2002; Mostl et al. 2002). Changes also exist in carcass bruising, as noted by Hoffman et al. (1998). A significant stress load imposed in connection with transportation to slaughter may also result in the cattle dying en route. We ascertained that the dairy cow death rate attributable to transportation is 0.038%. This level of mortality is relatively low and confirms a substantially higher resistance of dairy cows to transportation-induced stresses, in contrast to e.g. pigs, where the transportation-caused mortality in the Czech Republic is considerably higher (0.107% ± 0.013%), as reported by Večerek et al. (2006).

Dixit et al. (2001), Hoffman et al. (1998), and Villarroel et al. (2003) document the changes in the stress level indicators over long-lasting cattle transports. The rise in the number of deceased dairy cows with increasing distance demonstrates that shipments of long duration elevate the stress levels and lead to a higher mortality in dairy cows transported to slaughter, specifically from 0.013% for distances below 50 km to 0.183% for trips extending more than 300 km.

The discovery of a growing mortality trend in dairy cows transported to slaughter may be characterized as serious. These results prove that the conditions prevailing in the shipments of dairy cows have deteriorated. Worse transportation conditions are reflected in the lower quality of cow carcasses and the resultant meat, as mentioned by Broom (2003), Pištěková et al. (2004), Večerek et al. (2003), and Villarroel et al. (2003).

The statistics of dairy cow deaths may be suppressed by observing the requirements of proper transportation. Noteworthy among those are low-stress cattle-handling techniques (Grandin 1999), and stress-reducing conditions for slaughter-bound dairy cows as formulated for example by von Holleben et al. (2003) with respect to regrouping, loading, transport time, unloading, and lairage time. Wikner et al. (2003) also mention driving performance, handling during loading and unloading, and air quality inside the vehicle.

Úhyny dojnic při přepravě na porážku z pohledu přepravní vzdálenosti a ročního období

Výrazné nedodržování podmínek welfare při přepravě dojnic na jatky se může projevit zvýšenými úhyny zvířat při jejich přepravě.

Cílem práce bylo proto zjistit úroveň úhynů dojnic v souvislosti s přepravou na jatky, zjistit vliv přepravní vzdálenosti na úhyny dojnic, vliv ročního období na počty uhynulých dojnic a zjistit trend vývoje těchto úhynů.

Veterinární inspektoři v České republice v období let 1997 až 2004 sledovali úhyny dojnic v souvislosti s přepravou na jatky. Z celkového počtu 1 107 685 sledovaných kusů dojnic přepravených na jatky v České republice v období 1997 až 2004 uhynulo 418 kusů. Úroveň úhynů u dojnic byla zjištěna 0,038 %. Počet úhynů se však měnil v závislosti na přepravní vzdálenosti na jatky z 0,013 % při přepravě do 50 km až na 0,183 % při přepravě nad 300 km.

Při porovnání I. období (1997 až 2000) s II. obdobím (2001 až 2004) stoupl celkově počet uhynulých dojnic v souvislosti s jejich přepravou na jatky z 0,023 % na 0,057 % (index 2,51), tento rozdíl byl statisticky vysoce významný. Byl tak zjištěn dlouhodobý negativní trend vzestupu úhynů dojnic ve všech přepravních vzdálenostech.

Zjištěné výsledky dokládají poměrně nízkou citlivost dojnic na stresové zatížení způsobené přepravou na jatky projevující se úhyny v důsledku přepravy. Stoupající trend v úhynech dojnic v souvislosti s jejich přepravou na jatky je však varujícím zjištěním, které se týká welfare dojnic přepravovaných na jatky.

Acknowledgements

The work was supported by the Research Project No MSM6215712402 Veterinary aspects of food safety and quality.

References

BROOM DM 2003: Transport stress in cattle and sheep with details of physiological, ethological and other indicators. Dtsch Tierarztl Wochenschr **110**: 83-86

DIXIT VD, MARAHRENS M, PARVIZI N 2001: Transport stress modulates adrenocorticotropin secretion from peripheral bovine lymphocytes. J Anim Sci **79**: 729-734

GRANDIN T 1999: Easy tips for low stress cattle handling. Large Anim Pract **20**: 28-29

HOFFMAN DE, SPIRE MF, SCHWENKE JR, UNRUH JA 1998: Effect of source of cattle and distance transported to a commercial slaughter facility on carcass bruises in mature beef cows. J Am Vet Med Assoc **212**: 668-672

MORROW CJ, KOLVER ES, VERKERK GA, MATTHEWS LR 2002: Fecal glucocorticoid metabolites as a measure of adrenal activity in dairy cattle. Gen Comp Endocrinol **126**: 229-241

MOSTL E, MAGGS JL, SCHROTTER G, BESENFELDER U, PALME R 2002: Measurement of cortisol metabolites in faeces of ruminants. Vet Res Commun **26**: 127-139

MUDROŇ P, REHAGE J, SALLMANN HP, HÖLTERSHINKEN M, SCHOLZ H 2005: Stress response in dairy cows related to blood glucose. Acta Vet Brno **74**: 37-42

PALME R, ROBIA C, BAUMGARTNER W, MOSTL E 2000: Transport stress in cattle as reflected by an increase in faecal cortisol metabolite concentrations. Vet Rec **146**: 108-109

PIŠTĚKOVÁ V, OŠŤÁDALOVÁ I, SEDLÁKOVÁ J, TOMANOVÁ P, BEDÁŇOVÁ I 2004: Emergency slaughter of cattle due to immobility. Acta Vet Brno **73**: 533-538

VEČEREK V, KOZÁK A, MALENA M, TREMLOVÁ B, CHLOUPEK P 2003: Veterinary meat inspection of bovine carcasses in the Czech Republic during the period of 1995 - 2002. Vet Med-Czech **48**: 183-189

VEČEREK V, MALENA M, MALENA M jr., VOSLÁŘOVÁ E, CHLOUPEK P 2006: The impact of the transport distance and season on losses of fattened pigs during transport to the slaughterhouse in the Czech Republic in the period from 1997 to 2004. Vet Med-Czech **51**: 21-28

VERKERK GA, PHIPPS AM, CARRAGHER JF, MATTHEWS LR, STELWAGEN K 1998: Characterization of milk cortisol concentrations as a measure of short-term stress response in lactating dairy cows. Anim Welf **7**: 77-86

VILLARROEL M, MARIA GA, SANUDO C, GARCIA-BELENGUER S, CHACON G, GEBRESENBET G 2003: Effect of commercial transport in Spain on cattle welfare and meat quality. Dtsch Tierarztl Wochenschr **110** : 105-107

VON HOLLEBEN K, HENKE S, SCHMIDT T, BOSTELMANN N, VON WENZLAWOWICZ M, HARTUNG J 2003: Handling of slaughter cattle in pre and post transport situations including loading and unloading on journeys up to 8 hours in Germany. Dtsch Tierarztl Wochenschr **110**: 93-99

WIKNER I, GEBRESENBET G, TOLO E 2003: Dynamic performances of cattle transporting vehicle on Scandinavian roads and behavioural response of animals. Dtsch Tierarztl Wochenschr **110** : 114-120

YAGI Y, SHIONO H, CHIKAYAMA Y, OHNUMA A, NAKAMURA I, YAYOU KI 2004: Transport stress increases somatic cell counts in milk, and enhances the migration capacity of peripheral blood neutrophils of dairy cows. J Vet Med Sci **66**: 381-387

ACTA VET. BRNO 2006, 75: 455-461; doi:10.2754/avb200675030455

Bioindicators of Plant and Animal Origin in an Ecosystem Evaluation

J. ŽÁK

Faculty of Education, Palacký University, Czech Republic

Received August 26, 2004
Accepted March 16, 2006

Abstract

Žák J.: Bioindicators of Plant and Animal Origin in an Ecosystem Evaluation. Acta Vet. Brno 2006, 75: 455-461.

The study had a threefold scientific objective: to perform a botanical survey of the area under study, to identify all plant species grazed by the European hare *(Lepus europaeus)* and to determine polychlorinated biphenyl (PCB) presence in hares using the screening test. The study area is characterized by intensive agricultural operations around the town of Senice in the Olomouc region. During the botanical survey in the agrocoenosis in 2001 and 2002, the author identified 62 species of herbs and grasses and 8 woody species. Of that total, 19 herbal and grass species and 6 woody species were suitable for consumption by the European hare. To determine the PCB concentrations, samples of plants, and the liver and muscle tissue from the front and hind extremities of the European hare were collected. PCB concentrations ranged from 0.0004 to 0.0007 mg in plants and 0.0001 to 0.0005 mg in the liver and musculature of hares.

Congeners, screening, natural vegetation, PCB, Lepus europaeus

The excellent physiochemical characteristics of polychlorinated biphenyls (PCB) (thermo-stability, photo-stability, chemical inactivity, excellent heat-transferring characteristics etc.) have led to their extensive use practically in all the areas of human activity. In the last decades, however, PCB present high priority environmental contaminants and they are ranked among persistent organic pollutants (POPs).

The alarming moment was the finding of PCB in cattle in the village of Haňovice between 1985 and 1990, a merger of a number of small cooperative farms in the region into a gigantic agricultural co-operative JZD Senice, a conversion of a large part of the biotope into arable land that led to a more intensive use of artificial fertilizers (200 kg/ha) and pesticides (8 - 12 kg/ha), and to an almost total disappearance of woody plant species from the agrocoenosis.

The possibility of using hares for environmental monitoring purposes has been studied by Bukovjan (1992). Relationships within the environment and suitable ecological conditions for animals were studied by Forman and Godron (1993). The issues of nutrition of the European hare in the meadow and the woody environments were dealt with by Homolka (1982, 1987). Findings from other countries in the fields of rearing, ethology and nutrition of the European hare were reviewed by Kučera (1988). Polychlorinated biphenyls were demonstrated in soil, water, sediments, and plant and animal organisms by Piskač et al. (1990). Effects of PCB on hormonal activity, metabolism and fertility disorders were reported by Roots (1995), Jurajda and Bernardová (1996), Liang et al. (1999), Manosa et al. (2001). PCB has been demonstrated in fish, birds and mammals by Vávrová et al. (2003). Raszyk et al. (1996, 1997) reported faeces and manure as a possible source of PCB in agricultural ecosystems. These data emphasize the danger posed by BCPs and substantiate their inclusion among the 2A category (carcinogenic substances).

Materials and Methods

The study took place over a flat 300 hectare area situated 236 - 247 m above sea level in the central part of the Upper Moravian Vale, 20 - 23 km westward of the city of Olomouc (Czech Republic). Individual tracks of

Address for correspondence:
MVDr. Jiří Žák
Faculty of Education, Biology Department
Palacký University
Purkrabská 2, 772 00 Olomouc, Czech Republic

Phone: +420 585 635 181
E-mail: gabovaj@pdf.upol.cz
http://www.vfu.cz/acta-vet/actavet.htm

agricultural land in that region are large (100 - 200 hectares), with few bushes and trees, baulks or dirt roads. The most frequently grown crops include wheat, barley and, to a smaller extent, sugar beet. Synanthropic vegetation is found along sporadic dirt roads, ditches and in strips of land along railway tracks.

During the botanical survey performed by the author in the area in 2001 and 2002, two samples of plants weighing 1 kg were collected in the fields, in ditches along hard-surface roads and along railway tracks. The plant species making up the hares' diet were identified based on the signs of gnawing, autopsy of the digestive tract, specifically stomachs, of hares bagged in that area during the autumn hunting season. The autopsies were made according to methods used by the Veterinary Anatomical Pathology Section of the Veterinary Investigation Institute in Olomouc. Stomach content was studied under a magnifying glass (at 10 × magnification) and light microscope (CHK model, Made in Taiwan) (at 40 × magnification). PCB levels were determined from plant specimens, and muscle tissue and liver of hare bagged on the territory under investigation. Samples collected were stored in a freezer until they were handed over for analysis.

The PCB screening tests were performed by the State Veterinary Institute in Olomouc on 22 samples of liver tissue, hare leg muscle tissue and grass. PCB content was defined as a sum of indicator congeners. The number of samples was limited because hares were scarce, and hare hunting was subsequently banned. Before the autopsy was made, the condition, health and sex of the hares studied were examined.

All 22 autopsied hares were well nourished and showed no symptoms of diseases or pathological changes in their organs. Both sexes were approximately equally represented. Their age structure was not very good because hares aged 1 and 2 years predominated.

For PCB determination, extracted fat samples weighing between 0.1 and 0.2 g were used. Fat samples were dissolved in 2 ml of n-hexane and quantitatively transferred to a chromatography column filled with active florisil. The eluting agent was a 70 ml mixture of n-hexane and diethyl ether (94 : 6). The extract was evaporated by means of RVO and dissolved in 1 ml of eluent. If some of coextracts were not removed by the column chromatography, additional purification was performed by acid hydrolysis using sulphuric acid. One millilitre of the purified extract was then closed and prepared for the HRGC/ECD analysis. The instruments used included:

a) HP5 column
b) instrument for the determination of GC-ECD, model 5890, Series II
c) standards - certification mixtures EPA (BAKER)
d) extracted fat (reference material) manufactured by EHRENSTORFER
e) analytical equipment HRGC/ECD

Results

In the biotope studied, as in many other locations in the Czech Republic, the size of the population of a number of animal species has decreased markedly, and the European hare has been no exception. A particular attention was paid to the natural vegetation at the location studied, which, together with crops grown there, make up the hares' diet. In 2001 - 2002, the author spotted five European hares in the area, which is very few compared with the past.

In the region studied, 62 species of herbs and grasses and 8 woody species were identified (Randuška et al. 1985).

It was established that European hares consumed 29 of the total of 62 plant species identified in the area and 6 of a total of 8 wood plant species (Table 1). Most of the species were plants grown on agricultural land in the area studied. It follows from the plant species diagnostic that plants consumed by hares include very many that are poorly digestible, and are therefore not suitable as food for hares. Their food composition depends on the time of the year, types of crop on the fields, and the quality of wild grass (fine spring grass, mature old grass).

Tests for the presence of PCB were performed using plants and European hare tissues as bioindicators of environmental stress. Two samples of a variety of dried plants were used, each weighing 1 kg. Fresh samples of animal tissue, muscle and liver, were collected from bagged hares. The sum of 2 PCB congeners given in the table is in mg per kg of material.

Results of the tests in the muscle and liver tissues were statistically evaluated.

The mean values were 0.00033 and 0.00032 in the liver and muscles, respectively.

The standard deviations were 0.00015 and 0.00016 in the liver and muscles, respectively.

The coefficients of variations were 45% (liver) and 50% (muscles).

The average mean errors were 0.000032 (liver) and 0.000034 (muscles).

Table 1. Cultivated species, ruderal plants and other vegetation, woody species

Species	Family	Layer	Hares' diet
Achillea millefolium	Asteraceae	herbal	included
Aegopodium podagraria	Apiaceae	herbal	
Agrostis canina	Poaceae	herbal	included
Amaranthus retroflexus	Amaranthaceae	herbal	
Anthriscus silvestris	Apiaceae	herbal	
Arctium tomentosum	Asteraceae	herbal	
Arrhenatherum elatius	Poaceae	herbal	included
Artemisia vulgaris	Asteraceae	herbal	
Atriplex patula	Chenopodiaceae	herbal	
Atriplex sagittata	Chenopodiaceae	herbal	
Beta vulgaris	Chenopodiaceae	herbal	included
Ballota nigra	Lamiaceae	herbal	
Brassica napus	Brassicaceae	herbal	included
Bromus racemosus	Poaceae	herbal	included
Calamagrostis epigeios	Poaceae	herbal	
Carduus acanthoides	Asteraceae	herbal	
Convolvulus arvensis	Convolvulaceae	herbal	
Centauera jacea	Asteraceae	herbal	included
Chaerophylum album	Apiaceae	herbal	
Chaerophylum hirsutum	Apiaceae	herbal	
Chaenopodium album	Chenopodioceae	herbal	
Cichorium intibus	Asteraceae	herbal	included
Cirsium arvense	Asteraceae	herbal	
Dactylis glomerata	Poaceae	herbal	included
Elytrigia repens	Poaceae	herbal	included
Epilobium palustre	Onageraceae	herbal	
Equisetum arvense	Equisetaceae	herbal	
Euphorbia cyparissias	Euphorbiaceae	herbal	
Festuca pratensis	Poaceae	herbal	included
Festuca rubra	Poaceae	herbal	included
Galinsoga parviflora	Asteraceae	herbal	
Galium album	Rubiaceae	herbal	included
Geranium pratense	Geraniaceae	herbal	
Helianthus tuberosus	Asteraceae	herbal	included
Heracleum sphondylium	Apiaceae	herbal	
Hordeum vulgare	Poaceae	herbal	included
Hypericum perforatum	Hypericaceae	herbal	
Lamium album	Lamiaceae	herbal	
Lamium purpureum	Lamiaceae	herbal	
Leontodon hispidus	Asteraceae	herbal	
Linaria vulgaris	Scrophulariaceae	herbal	
Lolium perenne	Poaceae	herbal	included
Pastinaca sativa	Apiaceae	herbal	included
Phalaris arundinacea	Poaceae	herbal	included
Plantago lanceolata	Plantaginaceae	herbal	included
Plantago major	Plantaginaceae	herbal	included
Polygonum aviculare	Polygonaceae	herbal	included
Potentilla anserina	Rosaceae	herbal	
Potentilla reptans	Rosaceae	herbal	

Table 1 continued. Cultivated species, ruderal plants and other vegetation, woody species

Species	Family	Layer	Hare's diet
Rubus caesius	Rosaceae	herbal	included
Rumex crispus	Polygonaceae	herbal	included
Rumex sanquineus	Polygonaceae	herbal	included
Sinapis alba	Brassicaceae	herbal	included
Solanum nigrum	Solanaceae	herbal	
Solanum tuberosum	Solanaceae	herbal	included
Sonchus arvensis	Asteraceae	herbal	
Stellaria media	Caryophyllaceae	herbal	
Tanacetum vulgare	Asteraceae	herbal	
Taraxacum officinale	Asteraceae	herbal	included
Tripleurospermum inodorum	Asteraceae	herbal	
Triticum aestivum	Poaceae	herbal	included
Zea mays	Poaceae	herbal	included
Rosa canina	Rosaceae	bush	included
Sambucus nigra	Sambucaceae	bush	
Alnus glutinosa	Betulaceae	tree	
Cerasus avium	Rosaceae	tree	included
Fraxinus excelsior	Oleaceae	tree	included
Prunus cerasifera	Rosaceae	tree	included
Prunus domestica	Rosaceae	tree	included
Pyrus communis	Rosaceae	tree	included

PCB concentrations in liver, muscle and plant samples ranged from 0.001 to 0.0007 mg/kg. PCB concentrations in the samples were in compliance with Czech food standards. Tests performed by the Veterinary Investigation Institute in Olomouc showed that PCB concentrations in tissue and liver samples of the European hare bagged near Senice were low. There must therefore be other reasons than PCB for the decrease in hare populations in the fields of this region.

Discussion

A total of 62 grasses and herbs and 8 woody species were identified. It was established that European hares in the region consumed 29 of the grass and herbal species and 6 woody species (see Results), which is not enough.

Kučera (1988) made a list of species very suitable to excellent as food for the European hare. They are the following: *Thymus praecox, Petrosellinum crispum, Avena sativa, Triticum aestivum, Dactylis glomerata, Phleum pratense, Agrostis stolonifera, Festuca pratensis, Festuca rubra, Lolium perene, Pastinaca sativa, Medicago sativa, Trifolium campestre, Trifolium pratense, Trifolium repens, Trifolium medium, Capsulla bursa pastoris, Equisetum pratense, Brassica napus, Brassica rapa, Beta vulgaris, Daucus carota*.

In the 2001 - 2002 survey, the following six species from Kučera's list (1988) of plants consumed by the European hare were found in the area investigated: *Beta vulgaris, Lolium perenne, Festuca rubra, Dactylis glomerata, Pastinaca sativa, Triticum aestivum, Brassica napus, Agrostis canina, Festuca pratensis*.

The following 21 plant species consumed by the European hare in the biotope investigated do not appear on Kučera's list (1988): *Arrhenatherum elatius, Polygonum aviculare, Bromus racemosus, Galium album, Plantago major, Plantago lanceolata, Hordeum vulgare, Achillea millefolium, Helianthus tuberosus, Rumex crispus, Rubus caesius, Centaurea jacea, Sinapis alba, Stellaria media, Taraxacum officinale, Rumex*

Table 2. PCB congeners in mixed plant samples and the liver and muscle tissues of European hare in mg/kg of material

Sample No.	Tissue tested	PCB content mg/kg	Sample No.	PCB content mg/kg
Sample 1	Mixed plants 1	0.0005		
Sample 2	Mixed plants 2	0.0007		
1	Liver	0.0005	12	0.0004
	Muscle	0.0003		0.0002
2	Liver	0.0005	13	0.0001
	Muscle	0.0005		0.0002
3	Liver	0.0001	14	0.0001
	Muscle	0.0002		0.0002
4	Liver	0.0006	15	0.0004
	Muscle	0.0005		0.0005
5	Liver	0.0004	16	0.0005
	Muscle	0.0006		0.0005
6	Liver	0.0004	17	0.0005
	Muscle	0.0003		0.0005
7	Liver	0.0002	18	0.0004
	Muscle	0.0001		0.0001
8	Liver	0.0004	19	0.0002
	Muscle	0.0005		0.0001
9	Liver	0.0003	20	0.0002
	Muscle	0.0003		0.0003
10	Liver	0.0001	21	0.0004
	Muscle	0.0001		0.0003
11	Liver	0.0002	22	0.0005
	Muscle	0.0003		0.0006

sanguineus, Elytrigia repens, Phalaris arundinacea, Cichorium intibus, Solanum tuberosum, Zea mays.

Reporting on the situation in southern Moravia, Homolka (1982) described 111 plant species that constituted the trophic range. Green parts of herbs and grasses, forage crops and weeds predominated (Meriggi and Verri 1990). In winter, hares also gnawed at woody species. Trophic offer in a balanced biotope consists predominantly of grasses (up to 78%) and includes woody species in winter (20 - 40%) (Homolka 1982, 1987). The number of plant and woody species consumed by hares in the area investigated is small (29 and 6, respectively). The monodiet may have a negative impact on the European hare population in the area.

The establishment of large tracks of agricultural land and the ensuing reduction in the number of crops grown there (3 types) had a major impact on the scope of plants suitable for consumption by hares. Frequent digestive tract disorders in hares as a consequence of one-sided diet have been reported by e.g. Hell and Nováková (1979).

The plants consumed by hares may transport PCBs to their organisms. Polychlorinated biphenyls are chlorine-based xenobiotic substances that have penetrated to all parts of the environment. They were first demonstrated by Jensen (1966) in his study of chlorinated insecticides. Since then, PCBs have been detected in soil, water, sediments, plants and animals (Piskač et al. 1990; Vávrová et al. 2003). PCBs are substances of oily consistency, resistant to photochemical or biological degradation. They are produced by controlled chlorination of biphenyl at the presence of ferric chloride as the catalyst. Its

derivatives, e.g. chlorinated triphenyls, chlorinated dibenzofurans and dibenzodioxines, may also be potentially life-threatening (Kannan et al. 1987; Safe 1994). The WHO classification ranks PCBs among 2A compounds, i.e. carcinogenic substances that might cause the disease mainly in the liver, digestive tract and the lymphatic system. PCBs have been reported to have mutagenic and teratogenic effects, to interact with enzymes, particularly those of the endoplasmic reticulum, and to influence the metabolism of live organisms. Because PCB is contained in coating materials used in silage bunkers and silos, it might have easily penetrated into silages. PCBs have a mutagenic action and a negative effect on vitamin A levels (Bukovjan et al. 1992). PCBs in aquatic ecosystems were found by Bazzanti et al. (1997). The issue of PCB residues in European hares in central Bohemia was examined by Bukovjan et al. (1992). PCB in dolphins was found by Cardellicchio (1995), in turtles by Corsolini et al. (2000). Piskač et al. (1990) monitored effects of PCB on the chicken organism. Vávrová et al . (2003) reported that small mammals living in the soil were good bioindicators of PCBs and heavy metals. In the Czech Republic, PCBs were first isolated in the environment and their residua in sediments and organisms reported by Vávrová et al. (2003).

Trpák (1984) reported that DDT, whose chemical structure is very similar to that of PCBs, was still influencing animal populations at the time. The disturbed homeostasis of the lowland area investigated led to disproportions in the propagation of predatory and herbivorous insects (aphids, flea beetles vs. ground beetles, etc.). That necessitated a more intensive use of pesticides, mainly insecticides and rodenticides with toxic and cumulative effects (DDT, HCH, Endrin), which caused acute and chronic poisoning and the death of animals, including European hares serving as bioindicators, fertility disorders and tumour growths.

Examinations of samples for PCB showed that its values were within the norm. In a comparison between results from the present study (see Table 2) and results of the examination of liver and muscle tissue samples of hares from central Moravia (lowland region around the town of Kroměříž) very similar PCB values were found, according to SVÚ Olomouc. The values found in about 20 samples ranged from 0.0001 to 0.0007, i.e. they were approximately the same as the levels found in the area near Senice na Hané.

It is clear that the standard deviation is large. The tests have shown that PCB concentrations in dried mixed plant samples were higher than in animal tissues. PCB concentrations in muscles and liver were balanced, with slightly higher values found in the liver. The coefficient of variation is high, which means that the values measured are very variable.

The aim of the study was to determine all the plants growing in the area investigated, identify all plant species grazed by the European hare and to determine PCB presence in European hare using the screening test. The study was the first botanical study made in the area, and plant species that are consumed by hares and could participate in PCB transfer were identified.

Hodnocení ekosystému na podkladě použití bioindikátoru rostlinného a živočišného prostředí

V práci byla nastíněna situace chemické zátěže BCP a spektra rostlin vhodných pro zajíce polního jako potrava v intenzivně zemědělsky obdělávané krajině u obce Senice na Hané, okr. Olomouc. V roce 2001 byl v této agrocenóze proveden botanický průzkum. Bylo zjištěno 60 druhů bylin a 9 druhů dřevin. Dále byly stanoveny druhy rostlin, které jsou pro zajíce polního poživatelné. Jednalo se o 21 druhů bylin a travin a 8 druhů dřevin. Byl proveden odběr vzorků rostlin, jater a svalu přední nebo zadní končetiny zajíce polního za účelem určení hladiny PCB. Pozitivním jevem je, že hodnota PCB je v normě (rostliny mají

hodnotu od 0,0004 do 0,0007 mg, sval a játra od 0,0001 do 0,0005 mg). Cílem práce bylo stanovení všech druhů rostlin lokality, zjištění druhů rostlin, které slouží jako potrava zajíce polního a formou screeningového stanovení prokázání PCB u zajíce polního. Stanovení PCB bylo provedeno metodou HRGC/ECD. Z vědeckého hlediska je článek přínosem v tom, že lokalita byla zkoumána po botanické stránce a byly vyzkoumány druhy, které zajíc přijímá. Nejbližší údaje jsou z lokality Litovel. Zjistila se možnost kontaminace PCB z rostlin do těla zajíce.

References

BAZZANTI M, CHIAVARINI S, CREMISINI C, SOLDATI P 1997: Distribution of PCB congeners in aquatic ecosystems: A case study. Environ Int **23**: 799-813

BUKOVJAN K, HOFFMAN V, BUKOVJANOVÁ E 1992: Chemical residues in hares: Occurrence of organochlorine compounds and PCB in adipose tissue from field hares in central Bohemia. Fleischwirtschaft **72**: 339-341

CARDELLICCHIO N 1995: Persistant contaminats in dolphins: An indication of chemical pollution in the Mediterranean Sea. Water Sci Technol **32**: 331-340

CORSOLINI S, ARIGI S, FOCARDI S 2000: Presence of polychlorbiphenyls (PCB) and coplanar congeners in the tissues of the Mediterranean Loggerheat turtle *Caretta caretta*. Mar Pollut Bull **40**: 952-960

DYKYJOVÁ D 1989: Metody studia ekologie. Academia, Praha, 690 p.

FORMAN RTT, GODRON M 1993: Krajinná ekologie. Academia, Praha, 583 p.

HELL P, NOVÁKOVÁ E 1979: Zajíc polní jako ukazatel vlivu průmyslových imisí na CHKO Český kras. Památky a příroda **8**: 506- 509

HOMOLKA M 1982: The food of *Lepus-europaeus* in a meadow and woodlands complex. Folia Zool **31**: 243-254

HOMOLKA M 1987: The diet of brown hare *(Lepus-europaeus)* in Central Bohemia. Folia Zool **36**: 103-110

JENSEN S 1996: Report of a new chemical hazard. New Scientist **32**: 612

JURAJDA P, BERNARDOVÁ I 1996: PCB in fish of the river Morava (Danube basin). A sampling approach. Folia Zool **45**: 77-86

KANNAN N, TANABE S, WAKIMOTO T, TATSUKAWA R 1987: Coplanar polychlorinated biphenyls in Aroclor and Kanechlor mixtures. J Assoc Off Anal Chem **70**: 451-454

KUČERA O 1988: Umělý chov zajíce. SZN, Praha, 304 p.

LIANG Y, PONK MH, SHUTES R, REVITT D 1999: Ecological risk assessment of polychlorinated bifenyl contamination in the Mai Po marshes nature reserve, Hongkong. Water Res **33**: 1337-1346

MANOSA S, MATEO R, GUITART R 2001: A review of the effects of agricultural and industrial contamination on the Ebro delta biota and wildlife. Environ Monitor Asses **71**: 187-205

MERIGGI A, VERRI A 1990: Population - dynamics and habitat selection of the European hare on popular monocultures in northern Italy. Acta Theriol **35**: 69-76

PISKAČ A, RUPRICH J, HALOUZKA R 1990: Effect of polychlorinated-biphenyls (PCB) on the chickens organism - effect of a short-time administration of high-doses of delor-103 on the concentrations of thyroxine, triiodothyroxine, sodium, potassium and calcium in blood-serum. Vet Med Czech **35**: 237- 246

RANDUŠKA D, SOMŠÁK L, HÁBEROVÁ I 1985: Barevný atlas rostlin. Vydavateľstvo Bratislava, nakladatelství Profil Ostrava

RASZYK J, GAJDUŠKOVÁ V, ULRICH R, JAROŠOVÁ A, ŠABATOVÁ V, SALAVA J, PALAC J 1996: Occurrence of chlorinated pesticides and polychlorinated biphenyls in the stable and outside stable environment of farms for pig fattening.Vet Med Czech **41**: 165- 171

RASZYK J, TOMAN M, GAJDUŠKOVÁ V, NEZVEDA K, ULRICH R, JAROŠOVÁ A, DOČEKALOVÁ H, SALAVA J, PALAC J 1997: Effects of environmental pollutants on the porcine and bovine immune systems. Vet Med Czech **42**: 313- 317

ROOTS O 1995: Organophosporine pesticides and polychlorinated - biphenyls in the ecosystem of the Baltic Sea. Chemosphere **31**: 4085-4097

SAFE SH 1994: Polychlorinated biphenyls (PCBs) environmental- impact, biochemical and toxical responses, and implications for risk assessment Crit Rev Toxicol **24**: 87

TRPÁK P 1984: Problémy ochrany zoogenofondu. Veterinární péče v chovech lovné zvěře 3

VÁVROVÁ M, GARGOŠOVÁ HZ, ŠUCMAN E, VEČEREK V, KOŘÍNEK P, ZUKAL J, ZEJDA J, SEBASTIANOVÁ N, KUBIŠTOVÁ I 2003: Game animals and small terrestrial mammals - suitable bioindicators for the pollution assessment in agrarian ecosystems. Fresen Environ Bull **12**: 165-172

Book Reviews

Theodor Hiepe, Richard Lucius, Bruno Gottstein: Allgemeine Parasitologie mit den Grundzügen der Immunologie, Diagnostik und Bekämpfung. Parey in MVS Medizinverlag Stuttgart, 1st Edition, 2006, 447 pages, 150 illustrations, 34 tables. ISBN 3- 8304-4101-0.

Parasitology has done a significant progress over the last years in the field of both veterinary and human medicine, which is due to the implementation and application of the most up-to-date methodologies of biochemistry, pharmacology, immunology, genetics, ultramiscroscopy, and molecular biology. All these aspects are reflected in the above mentioned publication co-edited by three prominent European parasitologists, namely Dr. Dr.h.c. mult. T. Hiepe and Prof. Dr. R. Lucius from the Humboldt University of Berlin, and Prof. Dr. R. Gottstein from the University of Bern, and 26 other notable parasitologists from various universities and parasitological institutes from German-spoken countries (Germany, Switzerland, and Austria).

It is the first comprehensive elaboration on general parasitology comprising the broad issue of the whole field, appropriately completed with practically applicable chapters on diagnostics and suppression of parasitoses. The book is divided into 12 separate chapters. The introductory chapter defines parasitism and parasite-host relationships (apathogenic and pathogenic forms of their co-existence), the spreading of parasites within the animal kingdom, and possible ways of their transmission. Similarly the parasite specificity is further defined, and the ways of their nutritional, metabolic and mechanical influence; environmental aspects of parasitic infection are defined as well. The second chapter characterizes the understanding of parasitism from biological and economic points of view, and the evolutional aspect of parasite-host relationships, and possible confrontational alternatives of their mutual strategies. The third chapter sums up sources of paleoparasitic information, including findings gathered from mummies, coprolites, sediments and textiles, and possible impacts on the formation of settlement, human evolution and animal domestication. Chapter four gives a historical outline of the development of knowledge comprising findings from the antique period (China, India, Greek, Roman, and Byzantine cultural époques), important parasite findings of the Islamic and Occidental culture in the Middle Ages, as well as nominally presented researchers of modern times. Sixteen portraits decorate the inner sides of the book cover. Chapter five deals with the phylogeny and classification of parasitic eucaryota and presents the newest detailed systematics of parasitic protozoa, helminths and arthropods with a focus on veterinary and medically significant species in vertebrates. The sixth chapter elaborates in detail on the essentials of the biology of parasitic protozoa, helminths and arthropods. It characterizes the general physique, organelles of its locomotion, reproduction, and developmental cycles. Further sections provide detailed descriptions of forms, functions, classification and development of basic systematic units of individual strains and inferior systems, including significant species and relevant genotypes (*T. spiralis*). For example, the importance of *Arthropoda* is evidenced by the fact that their representatives have inhabited the globe for more than 500 million years; and of more than 1 million known species, about 40% pests have fundamental importance for veterinary and human health, also as vehicles of more than 50 viral, bacterial and protozoal infections. Chapter seven broadly deals with the most important principles of parasite metabolism, and in detailed schemes it demonstrates biochemical reactions in the process of nutrient degradation, energy gaining, and biosynthesis of amino acids, lipids, nucleotides, and in antioxidant mechanisms. Chapter eight, immunobiology of parasitic infections, consists of a general part describing efector and evasion mechanisms, the basics

of immunity response and immunopathology, and it further elaborates on these mechanisms in the most researched protozoal, helmintic and arthropod infections. The ninth chapter characterises the parasite and the host as part of the ecosystem with a focus on their quantitative, space and time distribution and dependency on dietary and regulatory factors. The origin and spreading of parasitic disease in the host population is broadly dealt with in chapter ten. The essentials of parasitological diagnostics constitute the eleventh chapter, divided into human and veterinary parts and completed with black-and-white image documentation. The last chapter sums up current knowledge of an effective and economical procedure in suppression of parasitoses in humans and animals. The closing chapter sums up knowledge of the most up-to-date antiparasitic means for therapy, prophylaxis and metaphylaxis. It includes the issue of antiparasitic resistance and available vaccines against parasites. The book ends with links to websites on the phylogeny, taxonomy, classification and diversity of parasites, and with a comprehensive reference list.

K. Chroust

WHO Expert Consultation on Rabies,
WHO Technical Report Series, No. 931, 2005, 96 p.
ISBN -13 9789241209311

This book is a joint effort of several authors who participated in The WHO Expert Consultation on Rabies held in Geneva from 5 to 8 October 2004. This report should be considered the most up-to-date information on rabies prevention and control, and supersedes that of the eighth report of the WHO Expert Committee on Rabies.

The content of the report is divided into twelve chapters followed by six practical annexes including a guide for post- exposure prophylaxis, rabies vaccination certificate for humans, addresses of international institutions, international rabies certificate for animals, case records form and a description of Rabnet, a mapping system for human and animal rabies.

Following a brief introduction, the second chapter describes the current taxonomy of the rabies virus and its classification into genotypes. New isolates of the virus to be characterized as new genotypes are also listed in this part.

Third chapter deals with the pathogenesis and diagnosis of rabies. Whereas the pathogenesis of the disease only briefly mentions elementary knowledge, the diagnosis of rabies fills almost six pages and contains detailed procedures of laboratory diagnosis including transport of specimens, biosafety considerations, and techniques for *post mortem* as well as *intra vitam* diagnoses. This chapter of the report is not sufficient to set up routine diagnostic tests, but represents a helpful review of contemporary diagnostic procedures. I appreciate especially that molecular techniques for virus detection were included (even though very briefly) at the end of this chapter.

Chapters four and five provide the reader with information on the treatment of rabid patients, transmission of the disease via organ transplants and *post mortem* management of the bodies of patients who have died of rabies. Since fatal cases of human rabies do not occur in Europe, the significance of this chapter for the Czech reader is rather limited, unlike the part dedicated to the treatment of rabies by vaccination, which provides recent data concerning the advantages and disadvantages of active and passive vaccination. Safety and efficacy issues of different vaccines for pre- and post-exposure prophylactic use are discussed.

Canine rabies was eradicated in North America, part of Europe, Japan and some areas in South America. However, canine rabies is still widespread in many countries and territories, predominantly in the developing world. These countries are urged to adopt and establish systems of rabies reporting, and to adopt measures to control rabies in dogs as well as in

different species of wild animals. Planning, implementation and evaluation of these measures are discussed in the remaining chapters.

The last part of the report is dedicated to research considerations for the 21st century. Proposed research areas cover not only basic research in the field of pathobiology and epidemiology, but also national health policy, legislation and regulation. Although the presented publication is dedicated to a limited circle of researchers, diagnosticians and physicians, it can be highly recommended to veterinary students, graduates and their teachers.

V. Celer

Terrestrial Animal Health Code 2005.
Fourteenth Edition, Office International of Epizootics, Paris, 2005, 634 pages.
ISBN 92-9044-635-8

The structure of the Code is the same as of the previous issue reviewed in Acta Veterinaria Brno, 2005, 74 (1):161-163. All the comments are valid also for the new issue 2005. It continues to apply the antisanitary policy based on the fraudulent principle "Import risk analysis is preferable to a zero risk approach" supporting openly the export of non-healthy animals and non-pathogen-free animal products. The Code, including three hundred pages of annexes, has been consistently avoiding everything that would demand from exporting countries a full sanitary quality, e.g. no one word on the most important conditions for avoiding spread of all pathogens through international trade.

Veterinary import conditions for meat, meat products, eggs, milk, milk products and other products of animal origin, representing more than a half value of international animal trade, have been systematically missing. These products represent the most important factors of invisible pathogen spreading. The import conditions protecting against food-borne diseases are missing as well, and thus consciously the door is open for their mass and daily international and post-import countrywide spread due to speedy distribution, as irreparable disastrous consequences of the extremely holey Code. This "policy" is reflecting minimal or zero etiological investigations of animal products in exporting countries interested in "smooth trade" (no investigations = no pathogen discovery = easy export, regardless of the pathogens). The Hazard Analysis Critical Control Point (HACCP) avoiding the contamination only does not bloc the streams of pathogens initiated at animal farms.

The Code is characterized by several historically unique features unimaginable in any other international trade standard. The Code unilaterally favours the exporting countries that do not respect the interests of importing countries in consequent protection of animal and human health. The Code does not admit that importing countries should demand full sanitary quality of animal commodities and the guarantee for them; on the contrary, it imposes the duty to accept non-healthy animals and non-pathogen-free food of animal origin. Exceptionally, the importing country can require healthy animals or pathogen-free food only if this is "scientifically justified" in writing to convince the exporting country (if this perverted logic would be applied e.g. on motor cars, then the importing country could not require fully functioning cars without written "scientific justification" !?). Instability of this Code is reflected in publishing every year a new issue with many amendments without marking their emplacements. Relatively easy and unpunished export of diseases, thanks to the Code, has lead to a stop of almost all disease eradication programmes in exporting countries due to the loss of any economic motivation.

In the chapter "Certification procedures" the most important provision for guaranteeing full independence on producers and exporters of veterinary attests issuing persons is missing as well as provision for consistent government supervision of them; they can confirm what

they wish without any legal responsibility. The lack of effective control becomes a breeding ground for corruption and thus disease export. This can be documented by now incalculable thousands of disease export cases in spite of having "OIE international veterinary certificates". These have only information value and do not guarantee animal health or pathogen-free status. In some exporting countries almost all private practitioners as "accredited veterinarians" (often without special training and exams) have government rubber stamp for "official" international veterinary certificates.

The chapter "Evaluation of veterinary services", overfull of details having nothing to do with international trade, reflects the Code concept to avoid any duty of exporting countries to guarantee full sanitary quality of animals and their products. One cannot find a single word on the most important criteria for international trade such as the knowledge of real national occurrence of diseases, control/eradication results, active surveys, average size of animal populations and trade per one public service veterinarian, etc. in the exporting countries. The Code does not respect the difference of dependency on producers and exporters, and it consciously does not distinguish between government service and difficult-to-control private service. The reason for this is that the major exporting countries have often very weak government services unable to control effectively the disease situation, to monitor the exported commodities and to issue international veterinary attests themselves. Following the World Bank policy, the Code has been degrading the role of government services that are, in fact, the only organizations qualified to initiate and organise nationwide animal disease control, eradication and surveillance.

The chapter on risk assessment, containing absurd and unrealistic procedures in international trade practice, is deliberately omitting the main factor such as disease situation in the exporting countries. The chapter abuses the risk evaluation to "facilitate trade" at the expense of human and animal health in importing countries. This is similar to the World Trade Organization (WTO) "Agreement on the Application of Sanitary and Phytosanitary Measures ", a "work" of New Zealand, one of the major exporting countries that seems to dominate the OIE (now World Organisation for Animal Health) and its Code Commission. This country itself has obviously no good idea about animal health situation (in 2004, it reported the occurrence of 35 internationally notifiable diseases and on 31 diseases of them reported "no information available"). Similar situation is in most of the major exporting countries. The OIE intentionally has not included in its information system data on nationwide active surveys providing more reliable disease occurrence data than the only available data on *ad hoc* reported cases representing the "tip of the iceberg". How can the importing countries possibly assess the risk of disease introduction without information on real situation in the exporting countries?

The Code provisions are often not the result of scientific risk assessment, as repeatedly demanded from the importing countries. The case of paratuberculosis can serve as an example: The above-mentioned country declared in 2004 that this disease was no more a hazard; as a follow-up the Code specific protective import conditions disappeared and the whole page entitled "Paratuberculosis" was left empty (something unknown in international standards). One cannot believe that the delegates of all member countries accepted willingly this " no-condition" opening "officially" the way for mass global spreading of paratuberculosis. This example documents that the Code has nothing to do with real protection of health and that the importing countries have not a slightest chance to protect themselves against a small group of the most influential exporting countries. These dominate the OIE and abuse it for their unscrupulous egoistic interests and damage in an irreparable manner both animal and human health in the world but also the reputation of veterinary medicine. The OIE, after becoming a WTO "affiliated branch" in 1995 seems to readily service to business instead of health, ignoring the basic principle of medicine - *Primum non nocere!*

The OIE itself, in spite of repeatedly calling for “convincing scientific risk assessment” to be elaborated by the importing countries, has never carried out and presented to member country governments any analysis of the practical impact of the Code on disease spreading through international trade. The OIE even abolished the useful information system including disease import, obviously due to the fear to disclose the truth that it has been converted into an organization that consciously supports international spreading of the vast majority of communicable diseases, including almost all zoonoses. The previous very useful Code has been converted, thanks to irresponsible trickery with the “risk assessment” replacing original zero risk concept, into the most dangerous international document for animal health in the history of veterinary medicine.

More information is available at www.cbox.cz/vaclavkouba/oiecode.htm.

Prof. MVDr Václav Kouba, DrSc.