ACTA VET. BRNO 2022, 91: 339–346; https://doi.org/10.2754/avb202291040339

The effects of egg yolk-based and egg yolk-free diluents on the post-thaw quality of bull spermatozoa

Jan Vodička, Jan Pytlík, Martina Stádníková, Luděk Stádník, Jaromír Ducháček, Radim Codl, Zuzana Biniová

Czech University of Life Sciences Prague, Faculty of Agrobiology, Department of Animal Science, Prague, Czech Republic

Received April 12, 2022
Accepted October 10, 2022

Abstract

The aim of this study was to evaluate the effects of 3 different commercially available extenders – AndroMed® (soy lecithin-based), Optidyl® (with the addition of ionized egg yolk), and BULLXcell® (with the addition of fresh egg yolk) – on bull spermatozoa quality, which was evaluated using the parameters of spermatozoa motility, viability, plasma membrane damage, acrosome damage, and mitochondrial activity after thawing and during a 2 h long thermoresistance test. The spermatozoa quality indicators were appraised by computer-assisted semen analysis and a flow cytometer. Significant differences (P < 0.01) between bulls were registered in all indicators measured. The highest average values of spermatozoa total motility and viability were achieved using BULLXcell® extender (44.33%; 52.06%). Variances in comparing this extender with Optidyl® and AndroMed® were −0.83%, −2.64%; −8.33%, −9.51%. The differences found between the egg yolk-based diluents (BULLXcell® and Optidyl®) and AndroMed® were significant (P < 0.01). Therefore, the more valuable extenders for bull semen dilution were egg yolk-based extenders, which provided higher post-thaw spermatozoa survival and quality than the soy lecithin-based extender.

Insemination dose, sire, semen, extender, thermoresistance, spermatozoa survival

Preserving the fundamental abilities of sperm during the long and demanding process of insemination dose production can provide a higher level of pregnancy rates in cows. It also reduces the consumption of straws, shortens the calving interval, and improves the economic effectiveness of breeding itself. The main objective is to achieve a pregnancy rate with frozen semen that is similar to that of with fresh semen or simply with natural breeding (Mocé et al. 2010). The process of cryopreservation generally reduces the viability of semen, and damages all the structures of spermatozoa, thereby impairing fertilization ability (Ugur et al. 2019). During the production of insemination straws, the functional status of spermatozoa is affected by many factors, such as pH, osmotic pressure, and stress associated with temperature changes (Lessard et al. 2000; Watson 2000; Rehman et al. 2013; Parisi et al. 2014). These stress factors cause reactive oxygen species production and lipid peroxidation of the cell membrane, which affect spermatozoa unfavourably (Wang et al. 1997). The elimination of these negative effects is obtained with the addition of suitable cryoprotectants in bull semen extenders, by its interaction with bulls' individuality (Vera-Munoz et al. 2011), by the controlled process of cooling, and by the use of optimal freezing curves (Pena et al. 2011).

Currently, there is a wide variety of available bull semen diluents of diverse origins. The most conventional ones are egg yolk-based, while relatively newly developed diluents are comprised solely of plant components (Murphy et al. 2018). Plant-based extenders should provide a comparable quality of frozen insemination doses and, at the same time, eradicate the possible disadvantages of egg yolk-based diluents, such as disease transmission,

Address for correspondence:
Doc. Ing. Luděk Stádník, Ph.D.
Department of Animal Science
Faculty of Agrobiology, Food and Natural Resources
Czech University of Life Sciences Prague
Kamýcká 129, 165 00 Prague 6 - Suchdol, Czech Republic

Email: stadnik@af.czu.cz
Phone: +420 224 383 057
http://actavet.vfu.cz/

microbial risk, and difficulty in standardization (Aires et al. 2003; Yildiz et al. 2013). The existence of an extender with such features would provide a valuable contribution to the artificial insemination industry; however, there are ongoing concerns over reduced fertility when plant-based extenders are used in bull semen cryopreservation (Layek et al. 2016). While some studies approved comparable *in vitro* quality or fertility rates of insemination straws diluted in egg yolk-free extenders (Aires et al. 2003; Stradaioli et al. 2007; Miguel et al. 2008; Miguel-Jimenez et al. 2020), others stated superior outcomes in preserving the sperm's abilities and in the fertilizing potential of straws diluted in egg yolk-based extenders (Muiño et al. 2007; Crespilho et al. 2012; Crespilho et al. 2014).

The aim of this study was to evaluate the effects of using various types of commercially available bull semen extenders for cryopreservation – egg yolk-free AndroMed® (based on soy lecithin), Optidyl® (based on ionized egg yolk), and BULLXcell® (based on fresh egg yolk) – on insemination straws' quality appraised by sperm motility, viability, plasma membrane and acrosome lesions, and mitochondrial activity during a 2 h long post-thaw thermoresistance test (TT).

Materials and Methods

Semen collection

Semen collection took place at the Artificial Insemination Center (Hradišťko, Central Bohemian Region, Czech Republic) over the course of one year. The semen was repeatedly collected from a pre-selected group of Holstein bulls used for breeding (n = 4) of the same age, type of housing, and management. All the bulls were standardly used for commercial purposes. After standard semen collection using an artificial vagina, trained staff in the laboratory evaluated the input quality of every semen sample collected. The main indicators were volume (VOL, g), density of the spermatozoa (DEN, $\times 10^6$ mm^{-3}), and motility percentage (MOT, %). For further processing, a minimal concentration and spermatozoa motility (DEN 0.7×10^6 mm^{-3} and MOT 70%) was required.

Semen processing

The amount of each semen sample obtained (n = 44) was split into 3 equal parts, and each part was diluted in a different commercially produced extender, differing in the type of cryoprotectant used. The first selected extender was AndroMed® (Minitübe GmbH, Tiefenbach, Germany), a standardly used egg yolk-free extender containing an extract of soy lecithin and antibiotics (Gentamicin, Spectinomycin, Lincomycin, and Tylosin). The second was Optidyl® (Biovet, Fleurance, France), an extender containing ionized egg yolk, Tris, and antibiotics (Streptomycin, Penicillin, Lincomycin, and Spectinomycin). The third extender was BULLXcell® (IMV, L'Aigle, France), with a 20% addition of fresh egg yolk. The diluted semen was filled into straws (0.25 ml, IMV, L'Aigle, France), then slowly cooled to 4 °C and equilibrated for 2 h in a cooling box.

After the timed equilibration was complete, the doses were transferred into a freezer which was already cooled to 4 °C. A direct freezing method (Digitcool®; IMV Cryo Bio System, L'Aigle, France) was used for freezing. The diluted semen was frozen by using a 2-phase freezing curve (Doležalová et al. 2015) and then stored in a liquid nitrogen container.

Semen thawing and sample evaluation

The straws were thawed in a 38 ± 1 °C water bath for a period of 30 s and subsequently evaluated. Spermatozoa total motility (%) was assessed using the CASA (computer assisted sperm analysis) method (SCA® Production v. 5.3., MICROPTIC, Spain) with a phase contrast microscope (Eclipse E200, Nikon®, Tokyo, Japan) at a × 200–300 magnification whereby a minimum of five fields of view per each straw were evaluated (Tuncer et al. 2011). Furthermore, the flow cytometry evaluation was performed as described by Savvulidi et al. (2021) with a small modification in the total viability evaluation, as the viable sperm were assessed as cells with an intact plasma membrane and an intact acrosome. The CASA and flow cytometry evaluations were performed immediately after thawing, and then 1 and 2 h after thawing. During the TT, the samples were kept in an INB 400 incubator (Memmert GmbH, Schwabach, Germany) at 38 °C.

Statistical evaluation

The acquired data were analyzed using the statistical software SAS 9.3. (SAS Institute Inc., Cary, NC, USA). The generalised linear model (GLM procedure) was used for the evaluation of individual effects. The Tukey-Kramer method was used for the evaluation of differences of the least square means. The following model equation was used:

$$Y_{ijkl} = \mu + A_i + B_j + C_k + (AB)_{ij} + (AC)_{ik} + e_{ijkl},$$

Description:

Y_{ijkl} – dependent variable (spermatozoa motility, spermatozoa viability, plasma membrane damage, acrosome amage, high mitochondrial activity after thawing, i.e. 0, 60, and 120 min of TT [T0-T120]);

μ – average value of the dependent variable;

A_i – i class bull fixed effect (for spermatozoa motility: i = 1, n = 45; i = 2, n = 45; i = 3, n = 45; i = 4, n = 45; for flow cytometer evaluated parameters: i = 1, n = 90; i = 2, n = 90; i = 3, n = 90; i = 4, n = 90);

B_j – j class extender fixed effect (for spermatozoa motility: j = AndroMed®, n = 60; j = Optidyl®, n = 60; j = BULLXcell®, n = 60; for flow cytometer evaluated parameters: j = AndroMed®, n = 120; j = Optidyl®, n = 120; j = BULLXcell®, n = 120);

C_k - k class evaluation time fixed effect (for spermatozoa motility: k = 0, n = 36; k = 60, n = 36; k = 120, n = 36; for flow cytometer evaluated parameters: k = 0, n = 120; k = 60, n = 120; k = 120, n = 120);

AB_{ij} – interaction between fixed effect of the bull and chosen extender;

AC_{ik} – interaction between fixed effect of the bull and an evaluation time;

e_{ijkl} – residual error.

Significance levels of $P < 0.05$ and $P < 0.01$ were used to evaluate the differences among groups.

Results

The model equation was significant ($P < 0.001$). All effects described were significant ($P < 0.001$), and the evaluated interactions were confirmed to be significant at a $P < 0.05$ significance level. The detailed values from the CASA and flow cytometry evaluations are presented in Table 1. As evident from Table 1, bull IV preserved the highest total motility of spermatozoa throughout the TT. The differences from the other bulls were significant ($P < 0.01$). The highest values of viability during TT were also achieved by bull IV (52.62%), where differences from the other bulls (−5.17%; −6.58% and −6.69%) were found to be significant ($P < 0.01$). Table 1 further presents that bull IV and bull II recorded the highest mitochondrial activity during TT ($P < 0.01$). Bull IV achieved the lowest values of plasma membrane damage during TT ($P < 0.01$) and, together with bull III, the lowest values of acrosomal damage during TT as well ($P < 0.01$).

A gradual decrease of the total motility and mitochondrial activity was observed during the TT, as the average motility and mitochondrial activity decreased from 51.53% and 65.21% post thawing to 29.44% and 54.44% respectively, after 120 min of incubation ($P < 0.01$). As evident from Table 1, the least suitable extender was AndroMed®, which preserved all evaluated sperm indicators at the lowest values, with the exception of mitochondrial and acrosome damage, which were the highest. The differences in egg yolk-based extenders were significant in all measured qualities ($P < 0.01$).

Further interpreted results are shown in Table 2, which describes the evaluated interactions. Significant impacts of the inter-sire (bull's individuality) effect on the motility, viability, mitochondrial activity, and membrane damage of thawed sperm in different diluents were observed in the current study ($P < 0.01$; $P < 0.05$). Additionally, it was statistically proven ($P < 0.01$) that the egg yolk-based extenders achieved higher spermatozoa motility than egg yolk-free diluent in all bulls except bull I. There were significant differences between individual bull evaluation of post-thawed total spermatozoa motility and evaluation of the motility at the end of TT in bulls II, III, and IV ($P < 0.01$). The biggest drop in motility during the TT was achieved by bull II (27.77%, $P < 0.01$). No significant differences were found between individual bull spermatozoa viability in the T0 and T120.

Discussion

Successful spermatozoa cryopreservation depends on several interrelated factors, including the initial quality of the semen, the composition of the extender, the cryoprotectant, the cooling protocol, the packaging, the thawing rate, and the interaction of these components, as well as individual animal variation (Cooter et al. 2005; Andrabi 2007; Clulow et al. 2008). The refinement of insemination dose production is therefore still a ceaseless process

Table 1. The effect of sire, evaluation time, and diluent on a post-thaw computer assisted sperm analysis and flow cytometry variables of bull spermatozoa.

Effect	Level	Total motility	Viability	Mitochondrial activity	Plasma membrane damage	Acrosome damage
Bull	Bull 1	43.67 ± 0.95^{A}	47.45 ± 0.49^{A}	59.76 ± 1.21^{A}	$50.81 \pm 0.48^{A,a}$	31.63 ± 0.40^{A}
	Bull 2	40.44 ± 0.95^{A}	46.04 ± 0.49^{A}	65.82 ± 1.21^{B}	$52.73 \pm 0.48^{A,b}$	33.70 ± 0.40^{B}
	Bull 3	31.89 ± 0.95^{B}	45.93 ± 0.49^{A}	49.88 ± 1.21^{C}	$52.89 \pm 0.48^{A,b}$	29.50 ± 0.40^{C}
	Bull 4	49.11 ± 0.95^{C}	52.62 ± 0.49^{B}	66.93 ± 1.21^{B}	45.66 ± 0.48^{B}	29.70 ± 0.40^{C}
Evaluation time	0	51.53 ± 1.06^{A}	46.72 ± 0.42^{A}	65.21 ± 1.05^{A}	52.25 ± 0.42^{A}	25.52 ± 0.35^{A}
	60	42.08 ± 1.06^{B}	51.28 ± 0.42^{B}	62.15 ± 1.05^{A}	47.42 ± 0.42^{B}	29.96 ± 0.35^{B}
	120	29.44 ± 1.06^{C}	46.02 ± 0.42^{A}	54.44 ± 1.05^{B}	51.89 ± 0.42^{A}	37.92 ± 0.35^{C}
Dilutent	Andromed®	36.00 ± 0.82^{A}	42.55 ± 0.42^{A}	54.56 ± 1.05^{A}	56.16 ± 0.42^{A}	36.13 ± 0.35^{A}
	Optidyl®	43.50 ± 0.82^{B}	49.42 ± 0.42^{B}	67.20 ± 1.05^{B}	49.16 ± 0.42^{B}	29.16 ± 0.35^{B}
	BULLXcell®	44.33 ± 0.82^{B}	52.06 ± 0.42^{C}	60.03 ± 1.05^{C}	46.25 ± 0.42^{C}	28.09 ± 0.35^{B}

Results are expressed as least square means ± standard error. Different uppercase superscripts (^{A,B,C}) in columns (within each effect) indicate significance at $P < 0.01$. Different lowercase superscripts (^{a,b,c}) in columns (within each effect) indicate significance at $P < 0.05$.

of utmost importance, as about only 50% of spermatozoa recover after thawing, even under the most refined and controlled freezing conditions and modifications (Layek et al. 2016). The differences in sperm motility and other spermatozoa features during the TT test confirm the effects of sire individuality on the final fertilization capability of spermatozoa and can also correlate with genetic factors explaining inter-species and breeds, as well as individual differences (Thurston et al. 2002).

Our findings of spermatozoa motility changes during TT are in accordance with Beran et al. (2013) and Doležalová et al. (2016) who recorded the highest motility at the beginning of the TT immediately after the thawing of the straws. Alcay et al. (2015) in their study comparing post-thawing spermatozoa motility in extenders with the addition of fresh or lyophilized egg yolk found no significant differences between these extender variants, which is consistent with our findings.

Our results are also comparable to Muiño et al. (2007), Celeghini et al. (2008), Crespilho et al. (2012), and Singh et al. (2018), who detected that egg yolk-based extenders provide higher values of post-thaw spermatozoa motility compared to soybean lecithin-based extenders. Opposing findings were found by Kumar et al. (2015), who reported higher sperm motility in insemination doses diluted in plant-based extender rather than egg yolk-based diluents. In the study conducted by Murphy et al. (2018), no significant differences between the total sperm motility of insemination doses diluted in AndroMed® and BULLXcell® were reported. Contrastingly, Amirat et al. (2005) observed that egg yolk-based diluents negatively affected spermatozoa motility, probably due to substances that these extenders contain, whereas soy lecithin-based diluents preserved higher total sperm motility. With an increasing emphasis on biosecurity issues and on controlling disease with regards to international sperm shipment, egg yolk extenders have become suspect for facilitating the transmission of diseases. Therefore, the potential of a soybean-based extender replacing egg yolk extenders has been investigated by several researchers (Aires et al. 2003; Muiño et al. 2007; Miguel et al. 2008;

Table 2. The effects of interactions between bull, diluent, and evaluation time on a post-thaw computer assisted sperm analysis and flow cytometry variables of bull spermatozoa.

Effect	Level	Total motility	Viability	Mitochondrial activity	Plasma membrane damage	Acrosome damage
Bull * Diluter	Bull 1 * Andromed®	$42.33 \pm 1.64^{A,a}$	$39.72 \pm 0.85^{A,a}$	35.17 ± 2.09^{A}	59.20 ± 0.84^{A}	31.00 ± 0.69^{A}
	Bull 1 * Optidyl®	$45.00 \pm 1.64^{A,a}$	48.32 ± 0.85^{B}	60.43 ± 2.09^{B}	50.50 ± 0.84^{B}	28.10 ± 0.69^{A}
	Bull 1 * Bullxcell®	$43.67 \pm 1.64^{A,a}$	49.76 ± 0.85^{B}	$54.06 \pm 2.09^{B,a}$	$48.98 \pm 0.84^{B,a}$	$29.39 \pm 0.69^{A,a}$
	Bull 2 * Andromed®	$33.33 \pm 1.64^{B,b}$	40.76 ± 0.85^{A}	$54.86 \pm 2.09^{B,a}$	57.80 ± 0.84^{A}	$41.73 \pm 0.69^{B,a}$
	Bull 2 * Optidyl®	$43.00 \pm 1.64^{A,b}$	$48.74 \pm 0.85^{B,a}$	$65.23 \pm 2.09^{B,b}$	$49.59 \pm 0.84^{B,a}$	27.28 ± 0.69^{A}
	Bull 2 * Bullxcell®	$45.00 \pm 1.64^{A,b}$	$52.85 \pm 0.85^{C,b}$	$59.19 \pm 2.09^{B,b}$	$45.03 \pm 0.84^{C,b}$	$25.89 \pm 0.69^{A,b}$
	Bull 3 * Andromed®	$25.33 \pm 1.64^{B,C,c}$	49.35 ± 0.85^{B}	66.56 ± 2.09^{C}	$49.21 \pm 0.84^{B,a}$	33.36 ± 0.69^{B}
	Bull 3 * Optidyl®	$35.33 \pm 1.64^{A,D,b}$	$52.92 \pm 0.85^{C,b}$	$69.90 \pm 2.09^{C,a}$	$45.33 \pm 0.84^{C,b}$	29.06 ± 0.69^{A}
	Bull 3 * Bullxcell®	$35.00 \pm 1.64^{A,D,b}$	55.58 ± 0.85^{C}	$64.33 \pm 2.09^{B,b}$	42.46 ± 0.84^{C}	26.68 ± 0.69^{A}
	Bull 4 * Andromed®	$43.00 \pm 1.64^{A,a}$	40.35 ± 0.85^{A}	61.67 ± 2.09^{B}	58.43 ± 0.84^{A}	$38.44 \pm 0.69^{B,b}$
	Bull 4 * Optidyl®	$50.67 \pm 1.64^{E,b}$	47.70 ± 0.85^{B}	$73.26 \pm 2.09^{C,a}$	51.20 ± 0.84^{B}	32.22 ± 0.69^{A}
	Bull 4 * Bullxcell®	$53.67 \pm 1.64^{E,b}$	50.05 ± 0.85^{B}	$62.54 \pm 2.09^{B,b}$	48.54 ± 0.84^{B}	30.43 ± 0.69^{A}
Bull * Evaluation Time	Bull 1 * 0	$48.89 \pm 2.12^{A,a}$	46.04 ± 0.85^{A}	57.41 ± 2.09^{A}	$53.11 \pm 0.84^{A,a}$	24.24 ± 0.69^{A}
	Bull 1 * 60	43.33 ± 2.12^{A}	$48.67 \pm 0.85^{A,a}$	$50.80 \pm 2.09^{A,a}$	$50.22 \pm 0.84^{A,a}$	28.28 ± 0.69^{B}
	Bull 1 * 120	$38.89 \pm 2.12^{A,a}$	43.09 ± 0.85^{B}	41.45 ± 2.09^{B}	55.35 ± 0.84^{B}	$35.97 \pm 0.69^{C,a}$
	Bull 2 * 0	$54.44 \pm 2.12^{B,a}$	$46.83 \pm 0.85^{A,a}$	$61.83 \pm 2.09^{A,b}$	52.32 ± 0.84^{A}	23.30 ± 0.69^{A}
	Bull 2 * 60	41.11 ± 2.12^{A}	$51.19 \pm 0.85^{A,b}$	$60.15 \pm 2.09^{A,b}$	47.26 ± 0.84^{B}	30.74 ± 0.69^{B}
	Bull 2 * 120	$26.67 \pm 2.12^{C,b}$	$44.32 \pm 0.85^{B,b}$	57.29 ± 2.09^{A}	52.84 ± 0.84^{A}	40.86 ± 0.69^{D}
	Bull 3 * 0	$43.33 \pm 2.12^{A,b}$	49.61 ± 0.85^{A}	$71.58 \pm 2.09^{C,a}$	$49.12 \pm 0.84^{B,b}$	25.23 ± 0.69^{A}
	Bull 3 * 60	33.33 ± 2.12^{C}	56.05 ± 0.85^{C}	$71.24 \pm 2.09^{C,a}$	42.54 ± 0.84^{C}	28.42 ± 0.69^{B}
	Bull 3 * 120	17.22 ± 2.12^{D}	52.19 ± 0.85^{A}	57.98 ± 2.09^{A}	$45.34 \pm 0.84^{C,b}$	35.45 ± 0.69^{C}
	Bull 4 * 0	59.44 ± 2.12^{B}	$44.41 \pm 0.85^{B,b}$	$70.02 \pm 2.09^{C,a}$	$54.45 \pm 0.84^{A,b}$	29.31 ± 0.69^{B}
	Bull 4 * 60	$50.56 \pm 2.12^{B,b}$	49.22 ± 0.85^{A}	66.41 ± 2.09^{C}	$49.67 \pm 0.84^{B,a}$	$32.39 \pm 0.69^{C,b}$
	Bull 4 * 120	35.00 ± 2.12^{C}	$44.48 \pm 0.85^{B,b}$	$61.03 \pm 2.09^{A,b}$	$54.06 \pm 0.84^{A,b}$	$39.39 \pm 0.69^{D,b}$

Results are expressed as least square means ± standard error. Different uppercase superscripts ($^{A, B, C, D}$) in columns (within each effect) indicate significance at $P < 0.01$. Different lowercase superscripts ($^{a, b,}$) in columns (within each effect) indicate significance at $P < 0.05$.

Miguel-Jimenez et al. 2020). Aires et al. (2003) and Amirat et al. (2005) reported that soy lecithin-based extenders performed better in the spermatozoa quality indicators than egg yolk-based extenders in freezing semen from Holstein bulls. On the contrary, a higher percentage of viable spermatozoa cryopreserved in egg yolk-based extender compared to soy bean lecithin-based diluents were observed in the studies by Crespilho et al. (2012) and Singh et al. (2018). Akhter et al. (2010) compared extenders based on soy bean extract with egg yolk-based diluents by evaluating viability, acrosomal status, spermatozoa motility, and a 60–90 day non-return rate in Holstein bulls. No significant difference was found between the extenders in these indicators. The same results, related to non-return rate when extenders based on egg yolk or soy lecithin were used, were found by Murphy et al. (2018).

These propositions are in contradiction not only to our results, but also to Celeghini et al. (2008) and Crespilho et al. (2012), where samples of semen diluted with soy lecithin-based extenders reached the lowest spermatozoa motility, viability, plasma membrane intactness, and acrosomal and mitochondrial status post-thawing, which are preconditions for greater *in vivo* fertility (Thun et al. 2002; Veerabramhaiah et al. 2015). The protective phase of soy lecithin is apparently limited during cryoconservation, resulting in a reduction of spermatozoa viability post-thawing and during artificial insemination (Crespilho et al. 2012).

Reduction and differences observed in individual bull spermatozoa quality indicators depend partially on the extenders used for cryopreservation, indicating that compounds present in the extender affect post-thaw semen quality differently (Kumar et al. 2003; Beran et al. 2012). As evident from the current study, some sires' sperm quality did not differ, whether the sperm was diluted in an egg yolk-based or in a soy bean lecithin-based extender, while other bulls maintained improved spermatozoa features when diluted in egg yolk-based extenders. These findings are consistent with the conclusions of Beran et al. (2012), who confirmed the effect of bull individuality on post-thaw characteristics and spermatozoa quality.

In conclusion, extenders based on egg yolk exhibited a higher spermatozoa motility after insemination dose thawing and simultaneously achieved a higher proportion of viable spermatozoa, mitochondrial activity, and membrane intactness in comparison to egg yolk-free extender. From the obtained results we can clearly recommend using egg yolk-based extenders for bull cryopreservation. In all the evaluated indicators of semen diluted with various types of extenders, individual differences among bulls were observed. To maintain the highest level of fertilizing ability of manufactured insemination straws, it would be highly appropriate to choose the proper semen extender with respect to the individuality of the bulls.

Acknowledgements

This research was funded by the Ministry of Education, Youth and Sports of the Czech Republic ("S" grant), the Ministry of Agriculture of the Czech Republic (NAZV Project No. QK22010270) and the Czech University of Life Sciences Prague (SGS Project No. SV21-6-21320).

References

Aires VA, Hinsch KD, Mueller-Schloesser F, Bogner K, Mueller-Schloesser S, Hinsch E 2003: *In vitro* and *in vivo* comparison of egg yolk-based and soybean lecithin-based extenders for cryopreservation of bovine semen. Theriogenology **60**: 269-279

Akhter S, Ansari MS, Rakha BA, Andrabi SMH, Iqbal S, Ullah N 2010: Cryopreservation of buffalo (*Bubalus bubalis*) semen semen in Bioxcell® extender. Theriogenology **74**: 951-955

Alcay S, Berk Toker M, Gokce E, Ustuner B, Tekni Onder N, Sagirkaya H, Nur Z, Kemal Soylu M 2015: Succesful ram semen cryopreservation with lyophilized egg yolk-based extender. Cryobiology **71**: 329-333

Amirat L, Anton M, Tainturier D, Chatagnon G, Battut I, Courtens JL 2005: Modification of bull spermatozoa induced by three extenders: Biociphos, low density lipoprotein and Triladyl, before, during and after freezing and thawing. Reproduction **129**: 535-543

Andrabi SMH 2007: Fundamental principles of cryopreservation of *Bos taurus* and *Bos indicus* bull spermatozoa. Int J Agric Biol **9**: 367-369
Beran J, Stádník L, Bezdíček J, Louda F, Čítek J, Ducháček J 2012: Effect of sire and extender on sperm motility and share of live or dead sperm in bulls' fresh ejaculate and in AI doses after thawing. Arch Anim Breed **55**: 207-218
Beran J, Šimoník O, Stádník L, Rajmon R, Ducháček J, Krejcárková A, Doležalová M, Šichtař J 2013: Effect of bull, diluter and LDL-cholesterol concentration on spermatozoa resistence against cold shock. Acta Univ Agric Silvic Mendel Brun **61**: 1575-1581
Celeghini ECC, Arruda RP, Andrade AFC, Nascimento J, Raphael CF, Rodrigues PHM 2008: Effects that bovine sperm cryopreservation using two different extenders has on sperm membranes and chromatin. Anim Reprod Sci **104**: 119-131
Clulow JR, Mansfield LJ, Morris LHA, Evans G, Maxwell WMC 2008: A comparison between freezing methods for the cryopreservation of stallion spermatozoa. Anim Reprod Sci **108**: 298-308
Cooter PZ, Goolsby HA, Prien SD 2005: Preliminary evaluation of a unique freezing technology for bovine spermatozoa cryopreservation. Reprod Domest Anim **40**: 98-99
Crespilho A, Sá Filho, Dell'Aqua M, Nichi M, Monteiro G, Avanzi B, Martins A, Papa FO 2012: Comparison of *in vitro* and *in vivo* fertilizing potential of bovine semen frozen in egg yolk or new lecithin based extenders. Livest Sci **149**: 1-6
Crespilho AM, Nichi M, Guasti PN, Freitas-Dell'Aqua CP, Sa Filho MF, Maziero RR, Dell'Aqua JA Jr, Papa FO 2014: Sperm fertility and viability following 48h of refrigeration: evaluation of different extenders for the preservation of bull semen in liquid state. Anim Reprod Sci **146**: 126-133
Doležalová M, Stádník L, Biniová Z, Ducháček J, Beran J 2015: The effect of freezing curve type on bull spermatozoa motility after thawing. Acta Vet Brno **84**: 383-391
Doležalová M, Stádník L, Biniová Z, Ducháček J, Stupka R 2016: Equilibration and freezing interactions affecting bull sperm characteristics after thawing. Czech J Anim Sci **61**: 515-525
Kumar S, Millar JD, Watson PF 2003: The effect of cooling rate on the survival of cryopreserved bull, ram and boar speermatozoa: a comparison of two controlled-rate cooling machines. Cryobiology **46**: 246-253
Kumar P, Saini M, Kumar D, Balhara AK, Yadav SP, Singh P, Yadav PS 2015: Liposime-based semen extender is suitable alternative to egg yolk-based extender for cryopreservation of buffalo (*Bubalus bubalis*) semen. Anim Reprod Sci **159**: 38-45
Layek S, Mohanty T, Kumaresan A, Parks J 2016: Cryopreservation of bull semen: evolution from egg yolk based to soybean based extenders. Anim Reprod Sci **172**: 1-9
Lessard C, Parent S, Leclerc P, Bailey JL, Sullivan R 2000: Cryopreservation alters the levels of the bull sperm surface protein P25b. J Androl **24**: 700-707
Miguel CT, Pérez-Garnelo S, Breña PB, Palasz A, De la Fuente J, Rodriguez A, Hidalgo C 2008: Freezing of semen from endangered asturiana de la montana bulls in zwitterionic lipid-based extenders. Reprod Fer Dev **20**: 161
Miguel-Jimenez S, Montserrat Rivera del Alamo M, Álvare-Rodríguez M, Hidalgo CO, Peña AI, Muiño R, Rodríguez-Gil JE, Mogas T 2020: *In vitro* assessment of egg yolk-, soya bean lecithin- and liposome-based extenders for cryopreservation of dairy bull semen. Anim Reprod Sci **215**: 106315
Mocé E, Purdy PH, Graham JK 2010: Treating ram sperm with cholesterol-loaded cyclodexrtrins improves cryosurvival. Anim Reprod Sci **118**: 236-247
Muiño R, Fernández M, Peña AI 2007: Post-thaw survival and longevity of bull spermatozoa frozen with an egg yolk-based or two egg yolk-free extenders after an equilibration period of 18 h. Reprod Domest Anim **42**: 305-311
Murphy EM, O'Meara C, Eivers B, Lonergan P, Fair S 2018: Comparison of plant- and egg yolk-based semen dilutents on *in vitro* sperm kinematic and *in vivo* fertility of frozen-thawed bull semen. Anim Reprod Sci **191**: 70-75
Parisi AM, Thomson SK, Kaya A, Memili E 2014: Molecular, cellular, and physiological determiants of bull fertility. Turkish J Vet Anim Sci **38**: 637-642
Pena FJ, Macias Garcia B, Samper JC, Aparicio IM, Tapia JA, Ortega Ferrusola C 2011: Dissecting the molecular damage to stallion spermatozoa: The way to improve current cryopreservation protocols? Theriogenology **76**: 1177-1186
Rehman FU, Thao C, Shah MA, Qureshi MS, Wang X 2013: Semen extenders and artificial insemination in ruminants. Veterinaria **1**: 1-8
Savvulidi FG, Ptáček M, Málková A, Stádník L 2021: Optimizing the conventional method of sperm freezing in liquid nitrogen vapor for Wallachian sheep conservation program. Czech J Anim Sci **66**: 55-64
Singh AK, Kumar A, Honparkhe M, Kaur S, Kaur H, Ghuman SPS, Brar PS 2018: Comparison of *in vitro* and *in vivo* fertilizing potential of buffalo bull semen frozen in egg yolk-, soya bean lecithin-, and liposome-based extenders. Reprod Domest Anim **53**: 195-202
Stradaioli G, Noro T, Sylla L, Monaci M 2007. Decrease in glutathione (GSH) content in bovine sperm after cryopreservation: comparison between two extenders. Theriogenology **67**: 1249-1255
Thun R, Hurtado M, Jannet F 2002: Comparison of Biociphos-Plus® and TRIS-egg yolk extender for cryopreservation of bull semen. Theriogenology **57**: 1087-1094

Thurston LM, Watson PF, Holt WV 2002: Semen cryopreservation: a genetic explanation for species and individual variation? Cryo-Lett **23**: 255-262

Tuncer PB, Sariozkan S, Bucak MN, Ulutas PA, Akalin PP, Buyukleblebici S, Canturk F 2011: Effect of glutamine and sugars after bull spermatozoa cryopreservation. Theriogenology **75**: 1459-1465

Ugur MR, Saber Abdelrahman A, Evans HC, Gilmore AA, Hitit M, Arifiantini RI, Purwantara B, Kaya A, Memili E 2019: Advances in cryopreservation of bull sperm. Front Vet Sci **27**: 268

Veerabramhaiah K, Rao A, Rao VH, Naidu K, Rao ST 2015: Efficacy of the Tris and Biociphos Plus Extender on the freezability of Punganue bull semen. Indian J Anim Reprod **32**: 1-4.

Vera-Munoz O, Amirat-Briand L, Bencharif D, Anton M, Desherces S, Schitt E, Thorin C, Tainturier D 2011: Effect of low-density lipoproteins, spermatozoa concentration and glycerol on functional and motility parameters of bull spermatozoa during storage at 4 °C. Asian J Androl **13**: 281-286

Wang Y, Sharma RK, Agarwal A 1997: Effect of cryopreservation and sperm concentration on lipid peroxidation in human semen. Urology **50**: 409-413

Watson PF 2000: The causes of reduced fertility with cryopreserved semen. Anim Reprod Sci **60-61**: 481-492

Yildiz C, Bozkurt Y, Yavas I 2013: An evaluation of soybean lecithin as an alternative to avian egg yolk in the cryopreservation of fish sperm. Cryobiology **67**: 91-94