The effect of a single dose of selenium with vitamin E on oxidative stress and fertilization indicators of ram sperm over the course of spermatogenesis

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Abstract

The aim of the experiment was to determine the effect of a single subcutaneous administration of selenium (Se) + vitamin E on the ejaculate volume, sperm count and viability, level of apoptosis and oxidative stress (OS) and Se concentration in ejaculates and blood of rams with respect to the time course of the spermatogenic cycle. The experimental group (EG; n = 6) was treated with a single injection of Selevit at a dose of 5 ml per animal (11 mg of sodium selenite and 125 mg of vitamin E/per animal). The control group (CG; n = 4) was treated in the same way, only with saline. Samples of blood and ejaculates were collected from each ram prior to application of Se and on the 1st, 14th, 26th, 38th, 50th and 62nd day after injection. Results showed that the Se concentration in the blood of EG was significantly higher only 24 h after application. Selenium concentration in the ejaculates of the EG was higher during the whole duration of the experiment (62 days), but significantly so only until day 14. The level of OS was significantly reduced on day 1, 14, and 62 after application of Selevit. There were no significant differences in the other analysed indicators. The results showed that one subcutaneous injection of Selevit had a positive effect on Se concentration and OS level in ejaculates, but was not sufficient to improve other monitored sperm quality indicators.

Spermatozoa, sperm quality, sodium selenite, rams, flow cytometry

As an integral part of glutathione peroxidase (GSH-Px), selenium (Se) is an irreplaceable component of the antioxidant system. GSH-Px activity has been reported in the semen of several species. If the Se content in selenoproteins is low, the possibility of fertilization is decreased (Dorostkar et al. 2012).

Reduced sperm production and poor sperm quality, including impaired motility with flagella defects localized primarily in the midpiece, have been detected in Se-deficient animals (Beckett and Arthur 2005). Vitamin E was found to decrease sperm abnormalities in the head of mice sperm and supplementation with Se and/or vitamin E improved the libido and semen characteristics in rams and bulls (Kendal et al. 2000; Raza et al. 2011; El-Sheshtawy et al. 2014).

Spermatogenesis is the process of cell proliferation and differentiation from a spermatogonial stem cell to adult sperm. These complex transformations occur in the seminiferous tubules of the mammalian testes and may proceed over an extended period of time, which is species specific (Hopper and King 2015). Therefore, it is important to take into account the effect of an applied substance not only shortly after its application, but also during the course of the whole cycle and after, which can last several weeks.

Most of the available works report positive effects of long-term supplementation by Se, with a preference for the organic form (Slowińska et al. 2011; El-Sharawy et al. 2017). For this reason, we decided to test the effect of a single-dose administraton of the inorganic form of Se on selected indices and the Se concentration in ejaculate and blood of rams with respect to the time course of the spermatogenic cycle.

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Materials and Methods

Ten adult Merino crossbreed rams aged 2–3 years and averaging 63 ± 4 kg body weight were divided into two groups. The experimental group, (EG; n = 6) was injected once subcutaneously with Selevit (BIOTIKA, Slovenská Ľupča, Slovakia) at a dose of 5 ml per animal (11 mg of sodium selenite corresponding to 5 mg of Se and 125 mg of vitamin E/per animal) according to manufacturer's instruction for a preventative dose. The control group, (CG; n = 4) was administered saline subcutaneously at a dose of 5 ml per animal.

Sample collection

Samples of blood and ejaculate were collected from each ram weekly during the period of three weeks before the application of Selevit. Semen was collected by electroejaculation and blood was obtained via jugular puncture. Selevit was applied subcutaneously six days after the third collection. Following blood and semen collections were performed on days 1, 14, 26, 38, 50 and 62 post injection with respect to the course of spermatogenesis and the duration of the sperm passage through the epididymis.

Analysis of blood and ejaculate samples

The semen was evaluated immediately after collection for volume, concentration, live and dead counts, and levels of apoptosis and oxidative stress (OS).

The Se concentration in blood and semen was determined using emission spectrometry with inductively coupled plasma on the optical emission spectrometer Optima 2100DV (PerkinElmer Inc., Shelton, USA) at the wavelength of 196.026 nm. The samples were mineralized by wet digestion in microwave laboratory systems Milestone MLS 1200 (MILESTONE s.r.l., Sorisole, Italy).

The vitamin E concentration in serum was analysed by the fluorometric method as described by McMurray and Blanchflower (1979).

Flow cytometry

The count, viability, apoptosis, and the level of OS of the sperm were determined by flow cytometry on a BD FACSCanto™ cytometer (Becton Dickinson Biosciences, San Diego, USA) equipped with blue (488 nm) and red (633 nm) lasers and 6 fluorescence detectors. The BD FACS Diva™ Software was used to analyse the obtained data.

Determination of sperm count

Counting beads, 123 count eBeads™ (eBioscience, ThermoFisher Scientific, Carlsbad, USA), were used for sperm counting according Evenson et al. (1993). For the analysis, semen was diluted 1:100 in sperm buffer (BTS powder, Jørgen Kruuse A/S, Langeskov, Denmark) and 25 µl of the sample was mixed with 100 µl of well-resuspended counting particles. The position of the sperm was delimited on the FSC-A (forward scatter-area) base dot blot graph opposite to SSC-A (side scatter-area) (i.e. the size of the cells opposite their granularity or inner complexity). This delimitation also included particle counts which have a high granularity. The particle position was then determined on the FITC-A (fluorescein isothiocyanate) dot blot graph opposite to PE-A (phycoerythrin) (Plate IX, Fig. 1). The cell count was determined according to the formula:

$$\text{Absolute number (sperm/ml)} = \frac{(\text{sperm count} \times \text{used particle volume})}{(\text{particles count} \times \text{used sperm volume})} \times \text{concentration of particles } (99 \cdot 10^4/\text{ml})$$

The numbers of sperm are expressed as $\log_{10}$ numbers/ml (mean ± standard deviation).

Determination of sperm viability

Sperm viability was determined by combined staining with propidium iodide (PI; Sigma Aldrich, St. Louis, MO, USA) and carboxyl fluorescein diacetate (cFDA; Sigma Aldrich, St. Louis, MO, USA) according to Ricci et al. (2002). This combination of fluorescent dyes allows us to distinguish between three types of cells: 1) living sperm cells emitting green fluorescence as a result of positive staining with cFDA; 2) dead sperm cells emitting red fluorescence by positive staining with PI; and 3) damaged sperm cells emitting fluorescence of both dyes (Dolník et al. 2019).

Staining procedure: 2.5 µl of 1 mM cFDA and 222.5 µl of PBS (phosphate buffered saline; MP Biomedicals, Illkirch-Graffenstanden, France) were added to 25 µl of sperm samples and diluted 1:100 in sperm buffer (BTS powder, Jørgen Kruuse A/S, Langeskov, Denmark). The sample was incubated for 15 min at 37 °C. Consequently, 1.5 µl of PI (1 mg/ml) was added and incubated for further 15 min at 37 °C. The sperm position was delimited on the FSC-A dot plot graph opposite to SSC-A and the viability was determined on the dot plot graph FITC-A opposite to the PE-A (Plate IX, Fig. 2).

Evaluation of sperm apoptosis

Thw commercial kit FITC Annexin V Apoptosis Detection Kit (BD Pharmigen, San Diego, USA) was used for evaluation of apoptosis according to Ricci et al. (2002). This kit contains propidium iodide (PI) and Annexin-V labelled with FITC. The evaluation method is the same as the determination of the viability, however, the position of the cells on the dot plot graph is FITC-A rather than PE-A (Plate X, Fig. 3).

Table 1. The effect of a single subcutaneous application of selenium (Se) + vitamin E on thover the course of the spermatogenic cycle and the passage of sperm through the epididymis. Results are expressed as mean ± standard deviation.

Indicator	Group	Time of collection (day)						
		0	1	14	26	38	50	62
Blood Se (µmol/l)	EG	2.10±0.37^{c}	4.40±0.70^{*b}	2.20±0.32^{a}	2.30±0.23	2.20±0.36^{c}	2.40±0.14	2.60±0.13
	CG	2.40±0.34	2.10±0.33^{a}	1.80±0.13^{b}	2.10±0.15	2.00±0.38	2.20±0.24	2.30±0.09
Semen Se (µmol/l)	EG	3.90±2.24^{a}	8.30±1.77^{*b}	6.10±1.16*	6.20±2.31	4.40±1.52	8.30±3.26	7.70±2.22
	CG	4.80±2.18	6.10±1.42	3.70±0.98	4.50±0.33	3.20±0.61	7.00±0.44	6.40±0.64
Ejaculate volume (ml)	EG	1.00±0.46	1.20±0.43	1.10±0.57	1.40±0.50	1.30±0.58	1.80±0.54	1.20±0.45
	CG	1.10±0.63	1.30±1.10	1.60±1.05	1.60±0.35	1.50±0.53	0.80±0.25	1.60±0.73
Number of sperm	EG	9.20±0.06	9.40±0.18	9.20±0.15	9.30±0.25	8.90±0.28	9.20±0.33	9.40±0.11
($\log_{10}$number/ml)	CG	9.40±0.24	9.30±0.16	9.00±0.46	9.00±0.33	9.00±0.14	9.00±0.11	9.40±0.33
Oxidative stress (%)	EG	29.70±9.55^{b}	4.00±3.21^{*a}	7.30±2.38*	18.00±5.80	16.30±5.11	26.20±5.58^{b}	1.70±1.21^{*c}
	CG	27.10±2.30^{a}	15.90±1.55	18.60±4.20	20.00±8.35	24.50±8.80	30.50±7.00	8.10±3.20^{b}

*Indicates significant difference between the experimental and control group at $P < 0.05$; different superscripts indicate significant differences between samplings: $^{a\text{-}b}$ $P < 0.05$ and $^{b\text{-}c}$ $P < 0.01$
EG – experimental group, CG – control group

Evaluation of oxidative stress level

Oxidative stress (OS) of the sperm cell membrane was assessed using the BODIPY C11 fluorescence dye according to Brouwers and Gadella (2003). Ten µl of 20 µM BODIPY 581/591 C11 solution of (4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid) (Molecular Probes, ThermoFisher Scientific, Oregon, USA) was added to 50 µl of sperm diluted 1:100. After 15 min of incubation at 37 °C, 1.5 µl of PI (1 mg/ml) was added and incubated for additional 15 min. After incubation, 200 µl of PBS were added and samples were analysed. In the evaluation, sperm position was demarcated at first, similarly to the determination of viability and consequently, the level of oxidative stress was evaluated on FITC-A dot plot graph for BODIPY versus PE-A for PI (Plate XI, Fig. 4).

Statistical evaluation of the results

All results obtained from this experiment were statistically evaluated in the GraphPadPrism Software using non-parametric Mann-Whitney test to compare treatment differences and Kruskal-Wallis test with supplementary Dunn's test to compare time differences.

Results

Selenium concentration in the blood of the EG was significantly higher ($P < 0.05$) only 24 h after Selevit injection (Table 1). Similarly, the concentration of vitamin E in serum was significantly increased only 24 h after application of Selevit (2.7 ± 0.69 µmol/l) compared to its levels before administration (1.4 ± 0.19 µmol/l; $P < 0.05$). In all subsequent samplings, vitamin E concentrations were comparable to pre-treatment values. Significantly higher values of Se concentration were found in the ejaculates of the EG compared to the CG on day 1 and 14 (in both collections $P < 0.05$) after Se supplementation. Within the time dynamics, a significant increase of the Se concentration in EG was seen between the pre-injection collection and day 1 post-injection ($P < 0.05$). There was no significant difference between the experimental and control group in the volume and density of the ejaculates (Table 1).

Higher values of oxidatively stressed sperm were found in the control animals. Significant differences between groups were reported on day 1 ($P < 0.05$), 14 ($P < 0.05$), and 62 ($P < 0.05$). A significant decrease of oxidatively stressed sperm was observed in the EG between the pre-injection collection and day 1 post-injection ($P < 0.001$). Then, a gradual increase of OS was recognised in this group until day 50. Another significant decline ($P < 0.001$) of the percentage of sperm damaged by oxidative stress was observed between days 50 and 62. (Table 1).

The administration of Se did not have a significant effect on the number of living and damaged sperm in the study. A significant decrease of the percentage of dead sperm was observed in the EG on day 26 compared to the CG ($P < 0.05$) (Plate XII, Fig. 5). The highest number of early-apoptic sperm (1.0–1.2%) was observed in the experimental group on day 38. However, a significant effect on the number of live/dead spermatozoa was reported only on day 14. There were higher concentrations of live sperm ($P < 0.05$) and significantly lower percentage of dead sperm in the EG compared to the CG ($P < 0.05$) (Plate XII, Fig. 6).

Discussion

During spermatogenesis, sperm cells pass through a number of developmental changes that result in morphologically functional sperm. The duration of spermatogenesis is species specific. According to Cardoso and Queiroz (1988), the duration of one cycle in a ram is 10.57 days and the time from the first spermatogonial mitoses to the release of sperm is 42.28 days. Subsequently, sperm cells are transported into the tail of the epididymis, which takes approximately 11 to 15 days. Here, sperm is stored in anabiosis until it is ejaculated. Spermatozoa are very sensitive cells, and therefore a number of exogenous and endogenous factors can affect them during spermatogenesis. Many authors have presented the positive effect of Se on the quality of ejaculate in many animal species (Ali et al. 2009; Dorostkar et al. 2012; Mahmoud et al. 2013). However, Audet et al. (2004) reported that addition of the inorganic form of Se to the feed did not improve the quality of ejaculates.

Our results showed that a single dose of Se + vitamin E injection caused an increase in the Se blood level only 24 h after application. Similarly, concentrations of vitamin E in blood serum were significantly increased only 24 h after application. Increased Se concentrations in sperm were maintained for at least 62 days. According to Smith et al. (1979), Se concentrations were higher in the reproductive apparatus (testes, sperm cells) than in other tissues and organs 23 days after intravenous administration of sodium selenite. At the same time, Smith et al. (1979) demonstrated that in the short period after Se administration, its concentration increased in seminal plasma but no increase of Se was observed in sperm. Therefore, we can conclude that the increased Se concentration 24 h after application was due to an excessive Se uptake by the accessory glands and Se was subsequently eliminated in seminal plasma. Increased Se concentrations on days 50 and 62 after the injection correspond with the increased incorporation of Se into sperm in the early stages of development, spermatogonia and primary spermatocytes. These conclusions are in accordance with the findings of Alabi et al. (2000). However, some authors (Smith et al. 1979) recorded an increase of Se concentration in the later developmental stages, namely secondary spermatocytes and spermatids, when the highest GPX expression was recorded (Puglisi et al. 2005). It was demonstrated by many authors (Smith et al. 1979; Bartle et al. 1980; Pond et al. 1983), that Se is incorporated into the developing spermatozoa in many animal species and has a function in the creation of structure in spermatozoa (Pallini and Bacci 1979; Alabi et al. 2000). These findings can explain the increase in Se concentration in the CG on days 50 and 62 in our study. This physiological mechanism is essential for the formation of normal morphological

structure of spermatozoa and the higher Se concentrations in the EG were due to increased Se supplementation. Similar results were demonstrated by Pond et al. (1983) who conclude that Se is incorporated into the developing spermatozoa but most likely before or during the spermatid stage. According to Olson et al. (2005), testes have a regulatory mechanism that can efficiently utilize Se to support spermatogenesis, even when deficient. It has been demostrated that the testes exhibit fairly higher Se concentrations compared to other tissues and organs (Ahsan et al. 2014). Behne et al. (1982) also reported that testes of Se-deficient rats maintained Se concentration within normal levels, although its concentration in other organs declined significantly.

Selenium is essential for the normal morphological structure of testes, but also for the development, maturation, and morphological and functional properties of sperm which affect the quality of the ejaculate (Shi et al. 2010). There were no significant differences in the ejaculate volume and sperm concentration in our study, which is consistent with the studies of Shi et al. (2010) and Piagentini et al. (2017). However, our results are not consistent with the results of other studies (Mahmoud et al. 2013; El-Sheshtawy et al. 2014) that recorded an increase in the ejaculate volume and density after application of a Se and vitamin E combination.

A significant effect on the viability of sperm in the Se-supplemented rams was detected only on day 26, when a significant decrease of dead sperm was observed compared to the control. Mahmoud et al. (2013) and El-Sheshtawy et al. (2014) detected an improvement in sperm viability after supplementation of Se and/or vitamin E. The positive effect of Se on sperm viability was also demonstrated *in vitro* (Dorostkar et al. 2012). However, Piagentini et al. (2017) and Ghorbani et al. (2018) did not find a significant effect of supplementation with either organic or inorganic Se on the number of living and dead sperm.

A large number of dead spermatozoa increases the formation of free radicals (FR), thus negatively affecting the ejaculate quality (Martí et al. 2003). According to Kumar et al. (2013) application of Se improves the antioxidant properties of seminal plasma by increasing the activity of antioxidant enzymes and reducing the level of lipoperoxidation. These findings are in accordance with the results of our experiment, where a significant increase of Se concentrations and a decrease of OS were observed in the EG in most collections compared to day 0. The lowest numbers of sperm damaged by OS in EG were recorded the first day after administration of Selevit and on day 62. The highest Se concentrations in the ejaculates were also recorded in these collections, which could be the reason for the decrease of the OS level in the samples. Reduction of oxidative stress on days 1 and 14 could be caused by the Se antioxidant protection in seminal plasma and the epididymal secretions. This is consistent with the results of Pond et al. (1983), according to who Se is not incorporated in adult sperm. The results on day 62 point to the incorporation of Se in the early stages of sperm development and the subsequent positive influence on the OS level in the samples. The positive effects of Se and vitamin E on the decrease of OS and lipid peroxidation were demonstrated by many authors (Horky et al. 2012; Hajalshaikh et al. 2015; Mudroň and Rehage 2018). Vitamin E is involved in the antioxidant defense of sperm and is one of the major membrane protective factors against reactive oxygen species and lipoperoxidation (Zubair et al. 2015). Therefore, the decrease of OS in the EG could be caused by the presence of vitamin E in the applied dose. A beneficial effect of vitamin E on antioxidant protection and sperm indice was reported by Azawi and Hussein (2013). However, according to Daramola et al. (2016), vitamin E did not have a positive effect on semen quality. El-Sheshtawy et al. (2014) found that a better effect of vitamin E was achieved when it was combined with Se. Free radicals cause lipid peroxidation, DNA damage, and apoptosis (Kothari et al. 2010). We observed a non-significant increase of sperm in the early phase of apoptosis in the EG during the

study. The percentage of sperm in the early phase of apoptosis was very low (up to 1.2%) throughout the experiment. According to Schulte et al. (2010) a certain level of apoptosis is needed to prevent sperm overproduction and to remove damaged sperm whose high levels negatively affect the ejaculate quality. Apoptotic sperm cells are removed mainly by Sertoli cells. Failure of this mechanism can lead to a high number of abnormal spermatozoa in semen and infertility.

Based on our results, we can assume that a single subcutaneous injection of Se + vitamin E is not sufficient to maintain higher concentrations of blood Se for a longer period. Although there was a long-term increase of Se concentration in ejaculates and a positive effect on the level of OS, no significant effect on the quality of ejaculates was observed.

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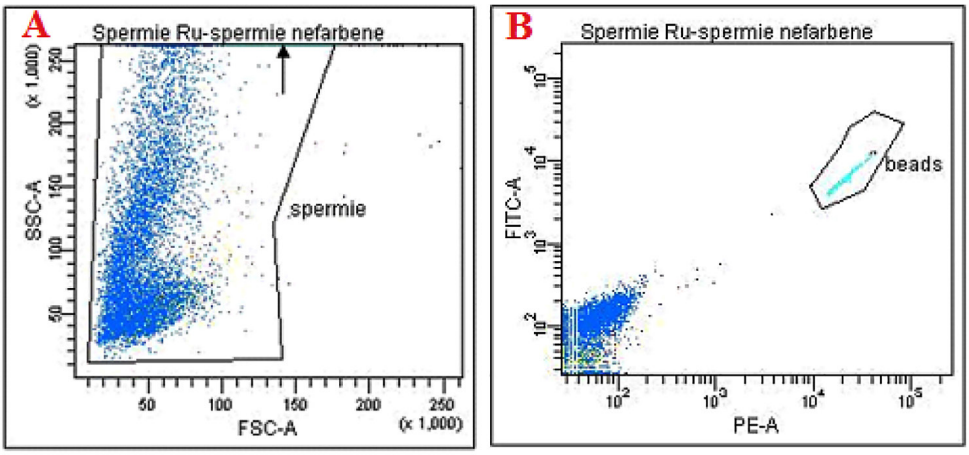

Fig. 1. Method to determine the number of sperm using 123 count eBeadsTM.

A: Sperm positioning on the basic dot plot graph FSC-A vs. SSC-A, a position of the particles is indicated by arrow. B: Position of fluorescence counting particles (light blue) on the dot plot graph FITC-A vs. PE-A. Sperm are not fluorescently dyed and they are in the lower left corner (dark blue).

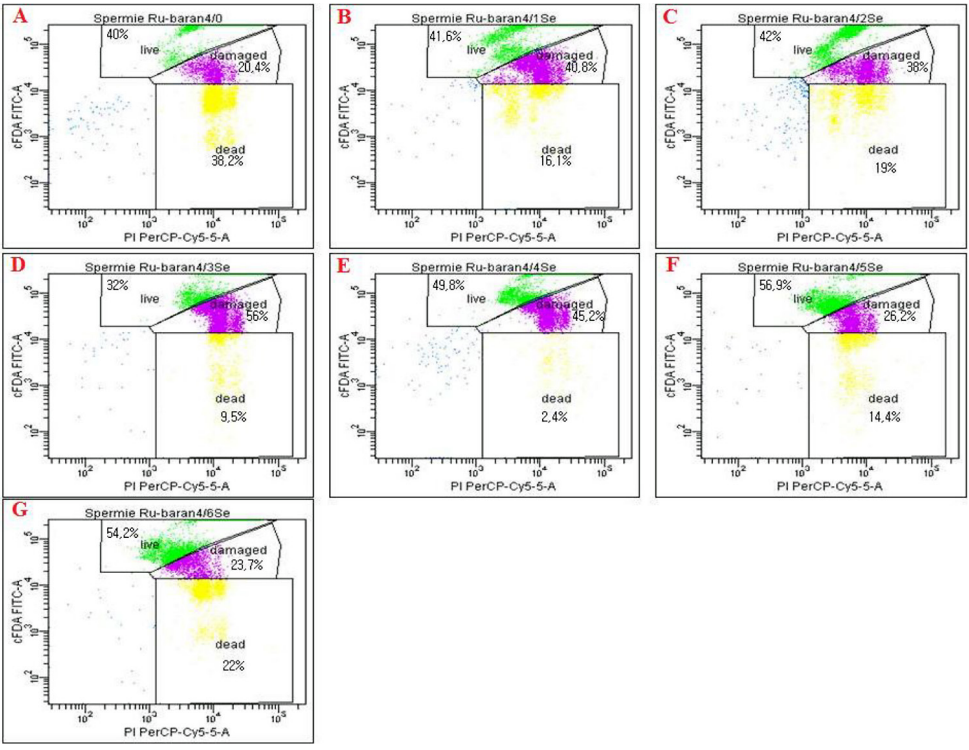

Fig. 2. Development of sperm viability from collection 0 to collection 6 (A, B, C, D, E, F, G) from one selected ram from the EG through dot plot graphs.

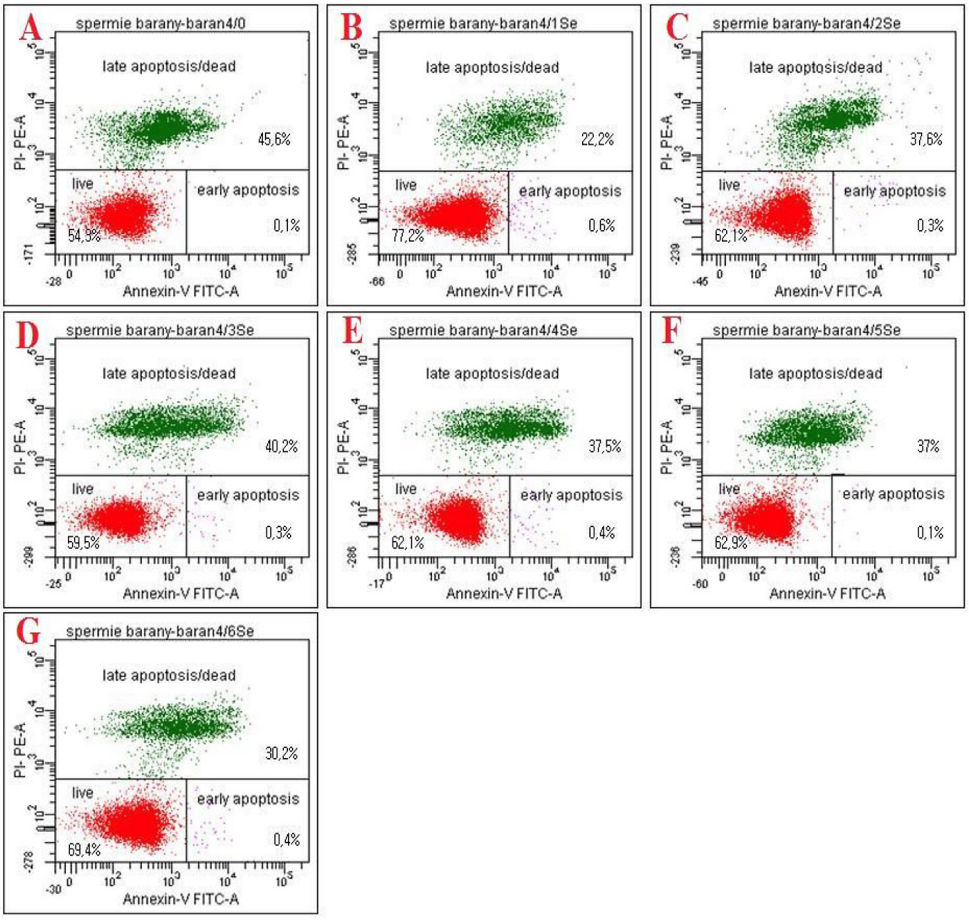

Fig. 3. Development of sperm apoptosis level from collection 0 to collection 6 (A, B, C, D, E, F, G) from one selected ram from the EG through dot plot graphs.

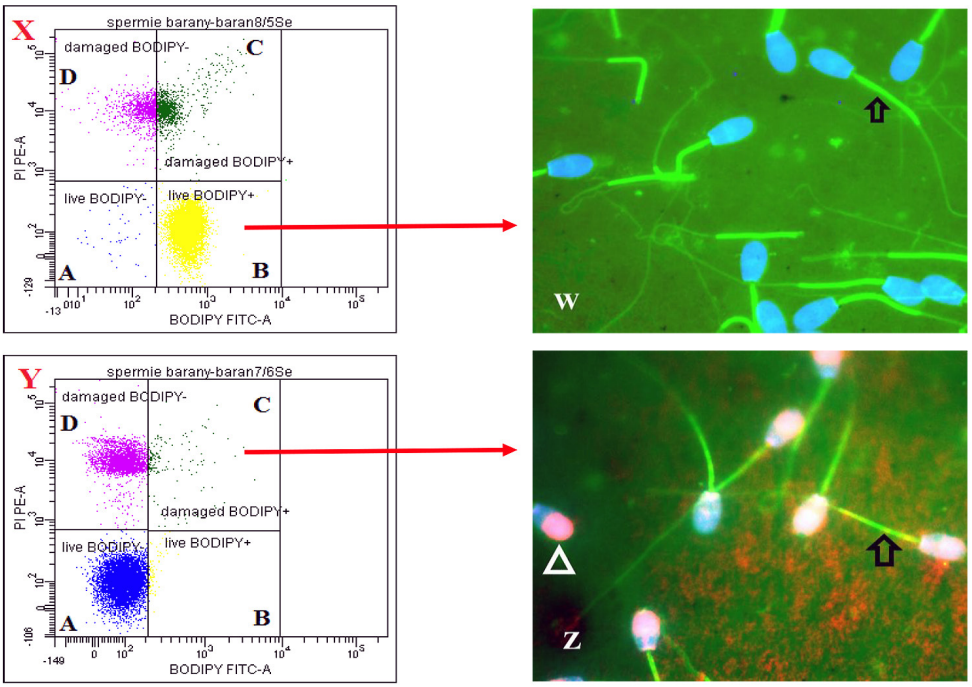

Fig. 4. Illustration of cytometric and microscopic examination of the lipoperoxidation level and damaging sperm using the dye C11-BODIPY 581/591 and PI. Y: High quality semen with low level of lipoperoxidation and X: semen with high level of oxidative stress. It can be distinguished in individual quadrants of the dot plot graph. A:

Live sperm without oxidative stress (blue). B: Live sperm with oxidative stress (yellow). C: Damaged sperm with oxidative stress (green). D: Damaged sperm without oxidative stress (purple).
On the images derived from the fluorescence microscope, the following is visible: W: live sperm with oxidative stress emitting green fluorescence (C11-BODIPY 581/591; marked with black arrow), Z: damaged sperm with oxidative stress emitting red (PI; marked with white triangle) and green (C11-BODIPY 581/591; marked with black arrow) fluorescence (source: own)

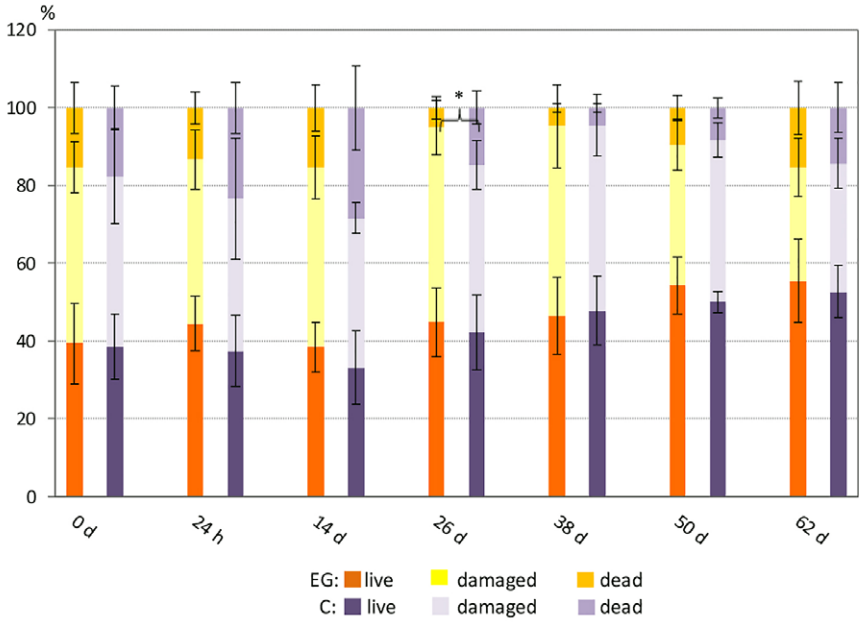

Fig. 5. The effect of a single application of selenium on the sperm viability of experimental (yellow-orange columns, n = 6) and control (purple columns, n = 4) group, expressed as percentages of live (darkest parts), damaged (brightest parts) and dead sperm (medium-coloured parts). Mann-Whitney test: * indicates significant difference between experimental and control group $P < 0.05$

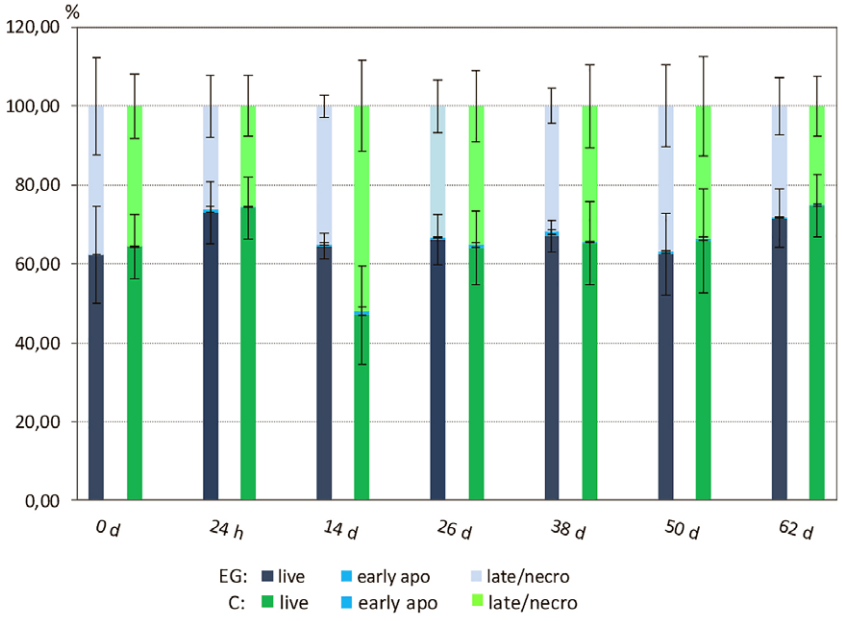

Fig. 6. The effect of a single application of selenium on the level of sperm apoptosis of the experimental (blue columns, n = 6) and control (green columns n=4) group, expressed as percentages of live sperm (darkest parts), sperm in the early phase of apoptosis, and sperm in the late stage or dead (necrotic) sperm (brightest parts).