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ULTRASTRUCTURAL ALTERATIONS IN SPERM HEADS UNDER INFLUENCE OF SEVERAL IMPLEMENTORS TO RAM SEMEN

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ABSTRACT

In our study we tested effects of chosen implementors on the acrosomal status of ram sperm heads on the basis of ultrastructure of sperm membranes and acrosome. Fresh sperm, diluted in a commercial diluent – Biladyl, was divided into five groups according to individual implementor: IGF-I, EGF, glutathione, caffeine and control (no additions). Individual sperm heads were classified as belonging to one of four categories according to the state of sperm membranes and acrosome: grade I – sperm with intact plasma membrane of the head and intact acrosome; grade II – sperm with waved or cracked plasma membrane; grade III – sperm with swollen or damaged acrosome; grade IV – sperm with pseudoacrosomal reaction formed by exocytotic vesicles and accompanied with a loss of acrosomal content. Our results show that the occurrence of sperm classified to the grade I was mostly increased when IGF-I or EGF were added to sperm samples. Oppositely, caffeine did not affect frequency of the grade I sperm, but increased the occurrence of grade III and IV sperm, i.e. induced pseudoacrosomal reaction. Glutathione did not influence the frequency of the grade I and II sperm but decreased the grade III sperm occurrence. In conclusion, IGF-I, EGF and glutathione were able to maintain the sperm membrane stability, which is important in increasing sperm fertilizing potential. Caffeine, although increased sperm motility, but caused damages in sperm head membranes and induced pseudoacrosomal reaction. Our results suggest that the categorization of sperm head into four morphological grades may serve to the evaluation of the membrane status of ram sperm.

Key words: ram; sperm; IGF-I; EGF; Glutathione; Caffeine; ultrastructure

INTRODUCTION

Membranes of ram sperm are rich for non-saturated fat acids and are sensitive to oxygen-induced damages caused by lipid peroxidation. Antioxidants of seminal plasma, which protect the sperm against oxidative damages induced by reactive oxygen species, are attenuated by dilution of the semen during preparation of insemination doses. Injuries of acrosomal membranes, like vesicle formation or alteration of permeability, can cause release and leakage of acrosomal enzymes and, as a consequence, decline fertilizing capacity of sperm. It was confirmed that the addition of glutathione, as an antioxidant, decreases blastocyst yield when added to sperm dose for fertilization (Earl et al., 1997). Lately it was documented that IGF-I (Vickers et al., 1999; Champion et al., 2002) and caffeine (Tatham et al., 2003) elevate sperm motility, which is resulted in higher fertilization rate of oocytes. A protein complex containing IGF-I purified from rabbit seminal plasma was used to investigate its effect on the capacitation and acrosome reaction of rabbit spermatozoa (Minelli et al., 2001). Recently it was reported, that several semen characteristics, including sperm concentration and motility, are influenced by plasma testosterone level (Kishk, 2008).

Using electron microscopy evaluation of the membrane and acrosomal integrity the sperm of different species was divided into several groups, from three to

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eight, according to the extent of morphological changes in the sperm head, membranes and the acrosome (Mahadevan and Trounson, 1984; Heath et al., 1985; Zibrín et al., 1987; Bwanga et al., 1991; Krogenaes et al., 1994; López et al., 1999; Okada et al., 2001; Nishizono et al., 2004; Sa-Ardrit et al., 2006). Above mentioned authors classified morphology of sperm basing on the changes in the membrane and acrosome of the apical part of the sperm head. In particular, Mahadevan and Trounson (1984) used 8 grades to classify morphological changes: (1) plasma membrane and acrosome are intact; (2) plasma membrane is swollen or damaged; (3) acrosome is swollen; (4) outer acrosomal membrane is damaged; (5) inner acrosomal membrane is damaged; (6) acrosome is lost; (7) acrosome and plasma membrane detached from the sperm head; and (8) acrosome-reacted sperm. In our opinion, for the evaluation of the status of plasma membrane, outer and inner acrosomal membrane as well as acrosome, a classification system involving 4 grades of morphology is sufficient. In our study we verified this grading system for the sperm head evaluation.

Electron microscopic study of the ultrastructure of frozen-thawed human spermatozoa was supported by clinical tests (Heath et al., 1985) and correlation was observed between the degree of damage, the changes in the ultrastructure and the fertilizing ability of the spermatozoa (Mahadevan and Trounson, 1984; Heath et al., 1985).

Our goal was to examine the effect of chosen implementors to ram semen (growth factors IGF-I and EGF, as well as caffeine and glutathione) on ultrastructural configuration of membranes and acrosomes of ram sperm heads.

MATERIAL AND METHODS

Sperm incubation in presence of implementors

Fresh ram semen (Lacaune breed) diluted in Biladyl diluent (Minitüb, Čeladice, Slovakia) was divided into 5 groups, and different implementors (all from Sigma-Aldrich, Germany) were added to sperm samples as follows: (1) IGF-I (10 ng/ml); (2) EGF (100 ng/ml); (3) caffeine (1 mg/ml); (4) glutathione (1 mg/ml); (5) control (no addition). Then sperm samples were incubated in a fridge at 4 °C for 5 days. At day 1 and day 5, the samples were picked up and placed into a thermostat at 39 °C for 40 min. Following the incubation sperm cells were washed in 0.15 M cacodylate buffer (pH 7.1-7.3) and embedded into agar to form pellets for electron microscopy (EM).

Preparation of sperm for EM analysis

Agar pellets of ram semen were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M cacodylate buffer (pH 7.1-7.3) for 1 h and then washed in cacodylate buffer. Afterwards samples were post-fixed in 1% osmium tetroxide in cacodylate buffer, washed in distilled water, dehydrated in acetone and embedded into Durcupan ACM (Fluka, Switzerland). The blocks of semen were cut into semithin sections (1–2 μm) using UC 6 Leica ultramicrotome (Leica Microsystems, MIKRO spol. s.r.o., Bratislava, Slovakia) and subsequently stained with toluidine blue. Thin sections (silver) were contrasted using uranyl acetate and lead citrate and viewed under a JEM 100 CXII electron microscope (Jeol, Japan) at 80 kV accelerating voltage.

Evaluation of sperm heads

Sperm heads were classified into 4 grades according to the membrane status (Fig. 1.): grade I – sperm with intact plasma membrane of the head and intact acrosome; grade II – sperm with waved or cracked plasma membrane; grade III – sperm with swollen or damaged acrosome; grade IV – sperm with pseudoacrosomal reaction formed by vesicles and with loss of acrosomal content.

Statistical analysis

At least 300 sperm heads per each group were analyzed. The frequency of each grade in all tested groups was statistically evaluated using Chi-square and Scheffe' test.

RESULTS

More than 70% of sperm cells on day 1 of the testing were distributed into the grade II (51%) and grade I (20.7%), whereas grades III and IV were represented by 28.4% of sperm cells (table 1.). At the end of the testing (day 5) most of sperm cells was distributed to the grade II (41.6%) and grade III (33.2%) of acrosomal morphology, whilst grades I and IV were represented by a lesser proportion of spermatozoa (table 1.)

From all implementors tested, a higher influence on the maintenance of intact sperm head plasma membrane and intact acrosome (grade I) on the day 1 was observed following addition of growth factors IGF-I and EGF ($P < 0.001$; table 1). Generally, IGF-I, EGF and glutathione caused higher distribution of sperm cells to first two categories - I and II (84%, 79% and 80% resp.) whereas lower distribution to last categories - III and IV (16, 21 and 20% resp.). Oppositely, caffeine caused lesser distribution of spermatozoa to grades I and II (41%), whilst a majority of sperm in the caffeine group (59%) was distributed to grades III and IV.

Table 1: Ultrastructural categorization of ram sperm heads after treatment with implementors

Implementor	Day	Distribution to the grades of morphology, n/ (%)			
		I	II	III	IV
Control	1.	43/ (14)	177/ (59)	63/ (21)	17/ (6)
IGF-I	1.	94/ (31)***	158/ (53)	38/ (13)	10/ (3)
EGF	1.	85/ (28)***	153/ (51)	51/ (17)	11/ (4)
Caffeine	1.	33/ (11)	90/ (30)***	97/ (32)**	80/ (27)***
Glutathione	1.	55/ (18)	186/ (62)	48/ (16)	11/ (4)
Totally day 1, n (%)		310/ (20.7)	764/ (50.9)	297/ (19.8)	129/ (8.6)
Control	5.	24/ (8)	141/ (47)	120/ (40)	15/ (5)
IGF-I	5.	33/ (11)	122/ (41)	102/ (34)	43/ (14)
EGF	5.	38/ (13)	135/ (45)	90/ (30)	37/ (12)
Caffeine	5.	33/ (11)	76/ (25)*	101/ (34)	90/ (30)***
Glutathione	5.	43/ (14.3)*	150/ (50)	85/(28.33)*	22/ (7.33)
Totally day 5, n(%)		171/ (11.4)	624/ (41.6)	498/ (33.2)	207/ (13.8)

*, **, *** - difference is significant compared to control at p<0.05, 0.01 and 0.001, resp. (Chi-square test)

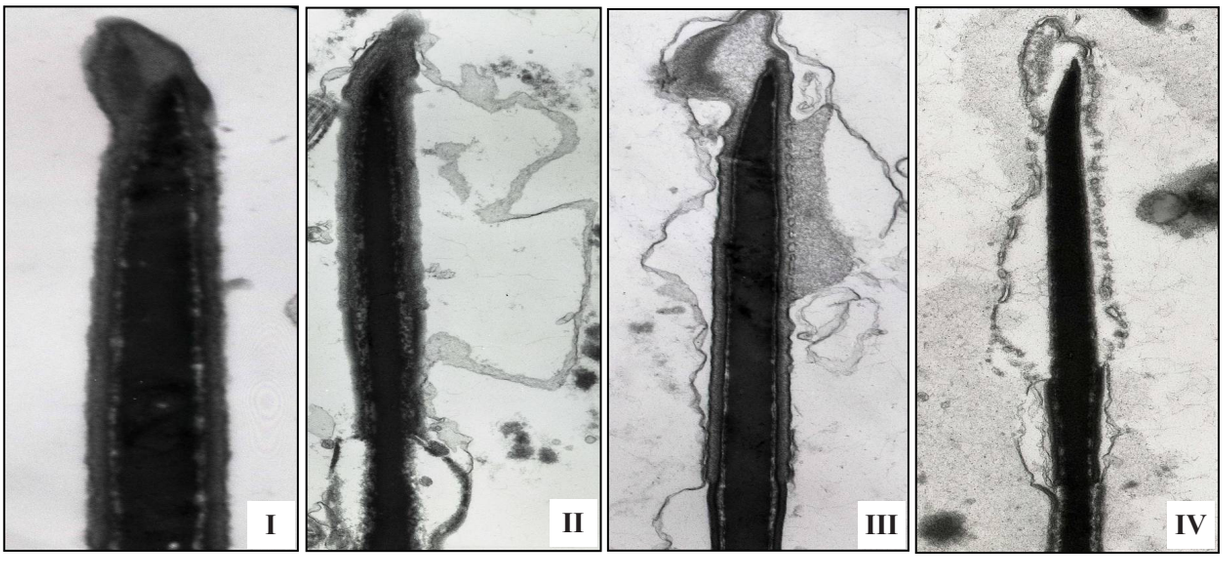

Fig. 1: Ram sperm heads classified to 4 grades according to state of membranes and acrosome:
I – grade I – sperm with intact plasma membrane of the head and intact acrosome;
II – grade II – sperm with waved and cracked plasma membrane;
III – grade III – sperm with swollen or damaged acrosome;
IV – grade IV – sperm with pseudo-acrosomal reaction formed by vesicles and accompanied with loss of the acrosomal content (I - x 38 000; II - and III - x 28 000; IV - x 20 000)

No differences between implementers were found on day 5 among all groups except glutathione for grades I and III (P<0.05 compared to control) and caffeine for grades II (P<0.05) and IV (P<0.001). Caffeine provided lower rate of the grade II but caused a higher frequency of the grade IV sperm compared to other groups. Generally, caffeine caused higher frequency of sperm belonging to grades III and IV, whilst grade I and II sperm was represented by a minimal occurrence (Table 1).

DISCUSSION

Undamaged cell membranes are necessary for the sperm viability. Membrane destabilization leads to functional capacitation, what enables sperm to fertilize the egg. However, when the sperm is deposited in female reproductive tract distanced from fertilization site, or maintained in vitro at standard temperatures, their longevity is shortened, what is resulted in the pre-matured sperm death (Mortimer and Maxwell, 2004). In the bull, boar and ram it was found that damage to the acrosome is the major cause of reduced fertility. The characteristic feature that distinguishes the high fertility from the low fertility groups is that fertile spermatozoa have intact acrosome and the low fertility spermatozoa have either damaged acrosomes or none at all. This confirms the notion that acrosome plays an important role in sperm survival and fertility.

We determined ultrastructural changes on the plasma membrane, acrosomal membrane and acrosome of ram sperm. We observed that the addition of several implementors caused membrane damages and release of acrosomal matter in different ways. Main consequence of these changes was a preterm release of acrosomal enzymes, what resulted in a decline of acrosomal matter density.

We characterized sperm fertilizing capacity using utrastructural characterization on the basis of four grades of acrosomal status. Sperm classified into first two grades are expected to have a higher fertilizing capacity compared to the sperm of grade III and IV, where damages of plasma and acrosomal membranes have already occurred.

Most studies indicate the efficiency of supplementation with various additives in order to preserve and increase cell membrane stability and sperm viability (Graham and Hammerstedt, 1992; Parks and Graham, 1992). Reactive oxygen species, such as H_2O_2 and other free radicals, have been known to cause decreased sperm capacitation and motility (Alvarez and Storey, 1989; Aitken et al., 1998). A more recent study by Lopes et al. (1998) suggested that oxidative stress causes DNA fragmentation in human spermatozoa. Moreover, when the function of sperm glutathione S-transferase was blocked, sperm motility, acrosome reaction and the ability to fertilize oocytes in vitro decreased, or were lost due to the membrane damage (Gopalakrishnan and Shaha, 1998). These facts suggest that the use of glutathione (GSH) during sperm preparation could be beneficial for membrane stabilization of sperm, and thus, helps in subsequent fertilization and development. GSH is a natural antioxidant present in both gametes but its level varies and plays an important role in protecting the all from oxidative damage (DeMatos and Furnus, 2000). An earlier study indicated that GSH has a protective effect against membrane damage in human sperm (Lenzi et al., 1994).

Our results showed that implementors IGF-I and EGF added to ram sperm, mostly elevated the occurrence of the grade I sperm and maintained the sperm membrane undamaged, what may result in higher fertilizing capacity, as was found by Vickers et al. (1999) and Champion et al. (2002). Under influence of these growth factors a proportion of the grade III and IV sperm was significantly decreased. The grade II sperm occurrence was lowered by both growth factors on the day 5 of the testing. Glutathione showed only moderate effect on sperm capacitation, as it did not influence the occurrence of grade I and II sperm, however lowered a percentage of the grade III sperm. Oppositely, caffeine did not influence a frequency of the grade I sperm, but decreased occurrence of the grade II sperm and elevated sperm occurrence in grades III and IV, i.e. led to acrosomal reaction. Park et al. (1989) tested effect of caffeine on the bull sperm in IVF and found that caffeine inhibits phosphodiesterase activity, what is resulted in rise of cAMP content and increase of sperm motility. Concerning caffeine, there are controversial data about its effects. Caffeine, although stimulates sperm motility and activity, but in our study, it impaired membrane integrity, induced vesicle formation and caused pseudoacrosomal reaction. Also, Mao et al. (2005) reported that caffeine may have caused hyper-motility of spermatozoa and resulted in polyspermy. A high incidence of polyspermic penetration in caffeine-containing IVF medium has been reported (Mao et al., 2005). Moreover, Tatham et al. (2003) suggested that caffeine can increase capacitation and fertilization, but at high concentrations in IVF medium is detrimental to embryonic development. Kihlman and Andersson (1987) reported that high concentration of caffeine causes DNA damage by inhibition of repair of chromosomal aberrations.

An efficiency of the addition of antioxidants - glutathione, alpha-tocopherol, acetate and aromex into ram semen was demonstrated by Sarlos et al. (2002). Authors have found that following addition of these substances the sperm motility was significantly increased and frequency of sperm abnormalities was decreased. The release of acrosomal enzymes may occur after addition of caffeine and also glutathione. Ram spermatozoa can have spontaneous acrosome reaction when incubated in a TRIS-buffered medium in vitro, but at low levels (Thompson and Cummins, 1985). Leakage of the acrosomal matter or acrosomal enzymes may occur between formed vesicles and also across seemingly undamaged membranes due to the change in their permeability. As known, ram sperm membranes are rich on unsaturated fat acids and are very sensitive to damages induced by lipid peroxidation. In this context it is possible that our tested implementors are

able to preserve sperm membranes against injuries.

In conclusion, growth factors (IGF-I, EGF) and glutathione, as implementors added to insemination doses, are able to improve sperm membrane stability and maintain their functional state and fertilizing ability. A categorization of sperm heads into four grades according to morphological status of membranes and acrosomes, using electron microscopy, may provide a basis for the evaluation of ram sperm viability.

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REFERENCES

AITKEN, R. J. – GORDON, E. – HARKISS, D. – TWIGG, J. P. – MILNE, P. – JENNINGS, Z. – IRINE, D. S. 1998. Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol. Reprod*., vol. 59, 1998, p. 1037-1046.

ALVAREZ, J. G. – STOREY, B. T. 1989. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res.*, vol. 23, 1989, p.77-90.

BWANGA, C. O. – EKWALL, H. - RODRIGUEZ-MARTINEZ, H. 1991. Cryopreservation of boar semen. III: Ultrastructure of boar spermatozoa frozen ultra-rapidly at various stages of conventional freezing and thawing. *Acta Vet. Scand*., vol. 32, 1991, p. 463-471.

DE MATOS, D. G. – FURNUS, C. C. 2000. The importance of having high glutathione (GSH) level after bovine in vitro maturation on embryo development effect of beta-mercaptoethanol, cysteine and cystine. *Theriogenol.*, vol. 53, 2000, p. 761-771.

EARL, C. R. – KELLAY, J. – ROWE, J. – ARMSTRONG, D. T. 1997. Glutathione treatment of bovine sperm enhances in vitro blastocyst production rates. *Theriogenol.,* vol. 47, 1997, p. 255 abstr.

GOPALAKRISHNAN, B. – SHAHA, C. 1998. Inhibition of sperm glutathione S-transferase leads to functional impairment due to membrane damage. *FEBS Lett*., vol. 422, 1998, p. 296-300.

GRAHAM, J. K. – HAMMERSTEDT, R. H. 1992. Differential efects of butylated hydroxytoluene analogs on bull sperm subjected to cold-induced membrane stress. *Cryobiol*., vol. 29, 1992, p. 106-117.

HEATH, E. – JEYENDRAN, R. S. - PEREZ-PELAEZ, M. – SOBRERO, A. J. 1985. Ultrastructural categorization of human sperm cryopreserved in glycerol and in TESTCY. *Int. J. Androl.*, vol. 8, 1985, p. 101-110.

CHAMPION, Z. J. – VICKERS, M. H. – GRAVANCE, C. G. – BREIER, B. H. – CASEY, P. J. 2002. Growth hormone or insulin-like growth factor-I extends longevity of equine spermatozoa in vitro. *Theriogenol.*, vol. 57, 2002, p.1793 -1800.

KIHLMAN, B. A. – ANDERSSON, H. C. 1987. Effects of caffeine on chromosomes in cells of higher eukaryotic organisms. Rev. Environ. Health, vol. 7, 1987, p.279-382.

KISHK, W. H. 2008. Interrelationship between ram plasma testosterone level and some semen characteristics. *Slovak J. Anim. Sci.*, vol. 41, 2008 (2), p. 67-71.

KROGENAES, A. - ANDERSEN BERG, K. – HAFNE, A. L. – ENGELAND, E. 1994. Membrane alterations in bull spermatozoa after freezing and thawing and after in vitro fertilization. *Acta Vet. Scand*., vol. 35, 1994, p.17-26.

LENZI, M. P. – GANDIRI, L. – LOMBARDO, F. – TERMINALI, O. – PASSI, S. - DOUDERO, F. 1994. Glutathione treatment dyspermia: effect on the lipoperoxidation process. *Hum. Reprod*., vol. *9,* 1994, p.2044-2050.

LOPES, S. – JURISICOVA, A. – SUN, J. G. – CASPER, R. F. 1998. Reactive oxygen species potential cause for DNA fragmentation in human spermatozoa. *Hum. Reprod*., vol. 13, 1998, p.896-900.

LÓPEZ, A. – SODERQUIST, L. – RODRIGUEZ-MARTINEZ, H. 1999. Sperm viability in ram semen diluted and stored in three different extenders. *Acta Vet. Scand*., vol. 40, 1999, p. 1-9.

MAHADEVAN, M. M. – TROUNSON, A. O. 1984. Relationship of fine structure of sperm head to fertility of frozen human semen. *Fertil. Steril*., vol. 41, 1984, p. 287-292.

MAO, J. – WU, G. M. – PRATHER, R. S. – SMITH, M. F. – CANTLEY, T. – RIEKE, A. – DIDION, B. A. – DAY, B. N. 2005. Effect of methyl-beta-cyclodextrin treatment of pig spermatozoa on in vitro fertilization and embryo development in the absence or presence of caffeine. *Theriogenol.*, vol. 64, 2005, p.1913-1927.

MINELLI, A. – LIQUORI, L. – COLLODEL, G. – LATTAIOLI, P. – CASTELLINI, C. 2001. Effects of the purified IGF-I complex on the capacitation and acrosome reaction of rabbit spermatozoa. *J. Exp. Zool. Angoli*, vol. 290, 2001, p. 311-317.

MORTIMER, S. J. – MAXWELL, W. M. C. 2004. Effect of medium on the kinematics of frozen – thawed ram spermatozoa. *Reproduction*, vol. 127, 2004, p. 285-291.

NISHIZONO, H. – SHIODA, M. – TAKEO, T. – IRIE, T. – NAKAGATA, N. 2004. Decrease of fertilizing ability of mouse spermatozoa after freezing and thawing in related to cellular injury. *Biol. Reprod.* Suppl., vol. 71, 2004, p. 973-978.

OKADA, A. – IGARASHI, H. – KURODA, M. – TERAO, K. – YOSHIKAWA, Y. – SANKAI, T. 2001. Cryopreservation-induced acrosomal vesiculation in live spermatozoa from cynomolgus monkeys *(Macaca fascicularis*). *Hum. Reprod*., vol. 16, 2001, p. 2139-2147.

PARK, C. K. – OHGODA, O. – NIWA, K. 1989. Penetration of bovine follicular oocytes by frozen-thawed spermatozoa in the presence of caffeine and heparin. *J. Reprod. Fertil*., vol. 86, 1989, p. 577-582.

PARKS, J. E. – GRAHAM, J. K. 1992. Effects of cryopreservation procedures on sperm membranes. *Theriogenol.*, vol. 38, 1992, p. 209-222.

SA-ARDRIT, M. – SAIKHUN, J. – THONGTIP, N. – DAMYANG, M. – MAHASAWANGKUL, S. – ANGKAWANISH, T. –

JANSITTIWATE, S. – FAISAIKARM, T. – KITIYANANT, Y. – PAVASUTHIPAISIT, K. – PINYOPUMMIN, A. 2006. Ultrastructural alterations of frozen-thawed Asian elephant (Elephas maximus) spermatozoa. *Int. J. Androl*., vol. 29, 2006, p.346-352.

SARLOS, P. – MOLNAR, A. – KOKAI, M. – GABOR, G. – RATKY, J. 2002. Comparative evaluation of the effect of antioxidants in the conservation of ram semen. *Acta Vet. Hung.*, vol. 50, 2002, p. 235-245.

TATHAM, B. G. – FEEHAN, T. – PASHEN, R. 2003. Buffalo and cattle hybrid embryo development is decreased by caffeine treatment during *in vitro* fertilization. *Theriogenol.*, vol. 59, 2003, p. 709 – 717.

THOMPSON, J. G. E. – CUMMINS, J. M. 1985. The effects of washing and protein supplementation on the acrosome reaction of ram spermatozoa in vitro. *Anim. Reprod. Sci.,* vol. 9, 1985, p. 75-86.

VICKERS, M. H. – CASEY, P. J. – CHAMPION, Z. J. - GRAVANCE, C. G. – BREIER, B. H. 1999. IGF-I treatment increases motility and improves morphology of immature spermatozoa in the GH-deficient dwarf (dw/dw) rat. *Growth Hormone & IGF Res*., vol. 9, p. 236-240.

ZIBRIN, M. – BELAK, M. – MESAROŠ, P. – GAMČIK, P. – TOMAJKOVA, E. 1987. The ultrastructure of frozen-thawed ram spermatozoa. *Z. Mikrosk. Anat. Forsch. Leipzich*., vol. 101, 1987, p. 904-912.