Journal of Microbiology, Biotechnology and Food Sciences

Špáleková et al. 2013 : 2 (Special issue 1) 1580-1591

REGULAR ARTICLE

RELATIONSHIP BETWEEN MOTILITY AND VIABILITY PARAMETERS OF FROZEN-THAWED BULL SPERMATOZOA

Eliška Špaleková [1, 2], Alexander Makarevich [1, 3], Elena Kubovičová [1, 3], Lucia Olexíková [1], Juraj Pivko [1]

[1] Animal Production Research Centre Nitra, Institute of Farm Animal Genetics and Reproduction, Hlohovecká 2, 951 41 Lužianky, Slovak Republic.

[2] Slovak University of Agriculture in Nitra, Department of Animal Physiology, Tr. A. Hlinku 2, 94901 Nitra, Slovak Republic.

[3] Research Institute for Cattle Breeding Rapotin, Ltd, 788 13 Vikyřovice, Czech Republic.

*Corresponding author: spalekova.eliska@gmail.com

ABSTRACT

The aim of this study was to determine relationship between parameters of spermatozoa motility (total motility - TM and progressive movement - PM) and viability of bull frozen-thawed spermatozoa (dead spermatozoa ratio, apoptotic spermatozoa ratio and plasma membrane integrity). Motility parameters were evaluated using computer-assisted semen analysis (CASA). Parameters of spermatozoa viability were analysed using fluorescent dyes PNA-FITC (plasma membrane), Yo-Pro-1 and propidium iodide (PI). All bulls (n=6) were divided into two groups. First group (n=3) A – better bulls with total motility after thawing over 40% and the second group (n=3) B – with total motility lower than 40%. It was observed significantly ($P<0.001$) higher TM and PM in group A. No significant differences in velocity parameters and ALH between the group A and B were detected. Occurrence of spermatozoa with disrupted membranes, dead/necrotic spermatozoa and apoptotic spermatozoa was significantly lower in the group A. Bulls in the group A showed significantly higher cleavage rate of embryos. These motility and viability characteristics are associated with a higher embryo cleavage rate in in vitro fertilization test.

Keywords : frozen-thawed spermatozoa, viability, motility

INTRODUCTION

Semen quality varies greatly from bull to bull. Semen from certain bulls may be of acceptable quality at collection but does not survive cryopreservation. The freezing and thawing process can adversely affect the nucleus, plasma, acrosome and mitochondrial membranes of spermatozoa (**Chatterjee *et al*., 2001, Aires *et al*., 2003, Amirat *et al*., 2004**). This generally can lead to loss of motility, swelling and the blebbing of the acrosomal membrane and disruption or increased permeability of the plasma membrane of spermatozoa (**Watson, 1976; White, 1993**). The detrimental damages can be prevented by suitable extenders and cryoprotectant additives (**Gil *et al*., 2003, Jeyendran *et al*., 2008**). Cryopreservation is associated with the production of reactive oxygen substances (ROS), which lead to lipid peroxidation of sperm membranes, resulting in a loss of sperm motility, viability and fertility (**Sariözkan et al., 2009**). This can adversely affect processes required for successful in vivo fertilization of the oocyte (**Bailey et al., 2000**).

The goal of our study was to determine potential relationship between parameters of spermatozoa motility (total motility, TM and progressive movement, PM) and viability of bull frozen-thawed spermatozoa (dead spermatozoa ratio, apoptotic spermatozoa ratio and plasma membrane integrity).

MATERIAL AND METHODS

Semen collection

The semen was collected once weekly from Holstein and Czech Fleckvieh bulls. Only fresh semen with required quality (minimum progressive motility 70% and spermatozoa concentration 0.7 x 10^6 ml^{-1}) was used for the subsequent processing of samples for observation according to common standards used for producing artificial insemination doses. The samples of the semen were diluted with AndroMed® diluent (Minitüb, Tiefenbach, Germany), a commercially produced extender containing soybean lecithin extract. Polyvinylchloride (PVC) straws (0.25 cm^3; IMV) were filled, cooled down to 4 °C and equilibrated for 90 min. Subsequently, the semen samples were frozen in a programmable

freezing device (IMV-Digitcool, L'Aigle, France) and then plunged into liquid nitrogen for storage.

Analysis of spermatozoa motility

Motility parameters were evaluated using computer-assisted semen analysis (CASA). The straws with frozen bull semen were thawed in a water bath at 37 ± 1°C for 1min. Motility parameters of the semen were analysed immediately after thawing at time intervals of 0, 0.5h or 2h, using CASA – Sperm Vision^{TM} 3.5 software. Between these time points the samples were incubated at 37 ˚C. A total motility (TM), progressive movement (PM) and selected movement parameters (curvilinear velocity VCL, average path velocity VAP, straight line velocity VSL, linearity LIN and amplitude of lateral head displacement ALH) were analyzed.

Fluorescent assays

Spermatozoa plasma membrane integrity was evaluated using fluorescently labelled lectin PNA-FITC (*peanut agglutinin;* Molecular Probes, Lucerne, Switzerland) in combination with propidium iodide (PI) which detected dead/necrotic spermatozoa in the samples (red signal) and DAPI (blue signal). Spermatozoa samples were unfixed; therefore, PNA marked only the spermatozoa with disturbed plasma membrane (green signal) while the spermatozoa with intact membranes remained unstained. Staining was done in a solution containing 20 μmol.l^{-1} of PNA-FITC and 5 μg.ml^{-1} PI in saline-FCS solutions for 20 minutes at room temperature. After the incubation, 4 μl of spermatozoa samples were mixed with 4 μl of Vectashield mounting medium with DAPI (H-1200, Vector Laboratories Inc., Burlingame, CA, USA). Samples were observed under a Leica fluorescent microscope (MIKRO spol. s r.o., Bratislava, Slovak Republic) with respective filters for green, red and blue fluorescence.

Apoptotic cells were detected using specific nuclear green fluorochrome, Yo-Pro-1 in combination with PI. The staining solution contained 5 μmol.l^{-1} of Yo-Pro-1 and 5 μg.ml^{-1} PI in saline-FCS solution. Spermatozoa with bright green fluorescent signal were regarded as apoptotic. The cells colored red were considered as dead or necrotic.

Analysis of spermatozoa fertilizing ability

Spermatozoa fertilization test was done using bovine prematured oocytes isolated from ovaries of cows provided by a local slaughterhouse. The recovered oocytes were matured during 24 h incubation in maturation medium 199 with glutaMax (Gibco Invitrogen, Auckland, New Zealand) supplemented with gonadotropins (FSH/LH 1/1 I.U., Pluset, Lab. Calier, Barcelona, Spain), sodium pyruvate, FCS (BioWhittaker) and gentamycine. Following maturation period, the oocytes were stripped out of the cumulus cells by vortexing and placed into fertilization drop (Fert-TALP medium) under mineral oil. The frozen-thawed spermatozoa were washed in a Sperm-TALP medium, resuspended in a fresh Fert-TALP medium and placed into a fertilization drop up to final concentration of 2 -3 x 10^6 spermatozoa per ml. Fertilization was performed in the incubator at 38.5°C in a humidified atmosphere with 5% CO2 during 20 h. Subsequently, the presumptive zygotes were cleaned off the excessive spermatozoa and the rest of cumulus cells by vortexing and transferred into B2 INRA culture medium (CCD Laboratories, Vernouillet, France) onto culture dish with previously prepared monolayer of BRL (Buffalo rat liver) cells. The zygotes were cultured at 38.5°C in a humidified atmosphere with 5 % CO_2 until evaluation. Cleavage rate of embryos at day 2 was recorded.

Statistical analysis

The experiments have been done in two replications. For spermatozoa motility analysis (CASA), 7 view fields per each group were evaluated (at least 750 spermatozoa per one experiment were counted). Average values were calculated from three measurements during the day. For fluorescent markers, more than 800 spermatozoa per each group were evaluated. The results were statistically evaluated by repeated measure ANOVA test and t-test using Sigma Plot 11.0 software.

RESULTS AND DISCUSSION

Values of total motility after thawing of all tested bulls ranged from 31.8 % (RAD 229) to 63.5% (BA 100). All bulls were divided into two groups according their total motility. First group A - better bulls (NEA 737, BA 100, NEA 532) which had total motility after thawing over 40% and the second group B – worse bulls (AMT 55, RAD 229, NEA 356)

which had total motility lower than 40%. Motility of 30% or greater is considered to be acceptable quality (**Person *et al.*, 2007**).

Total motility in the A group (better motile spermatozoa) was significantly higher (P<0.001) compared to the B group (worse motile spermatozoa). Similar tendencies were observed for progressive movement. The values of PM after thawing were ranged from 29.37 % (RAD 229) to 60.5% (BA 100). All bulls from the B group had significantly (P < 0.001) lower values of PM compared to A group (Fig 1). One common characteristics of cryopreserved spermatozoa is the decline in motility of the cells, that may cause relatively poor fertilising potential when are introduced into the reproductive tract at artificial insemination (**Watson, 2000**).

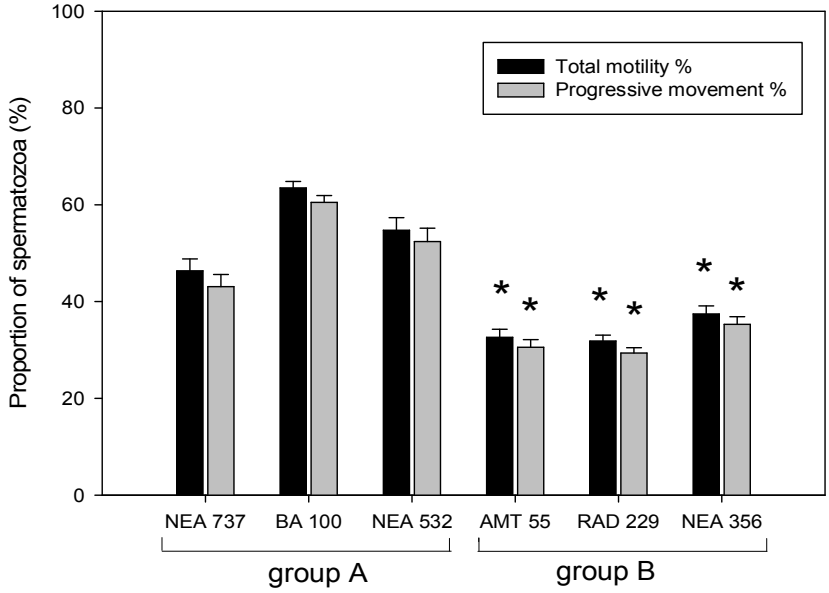

Figure 1 Total motility and progressive movement of frozen-thawed bull spermatozoa.
* - significant difference between the group A and B at P < 0.001

Except spermatozoa TM and PM, velocity parameters of spermatozoa were measured (VAP, VCL, VSL). No significant difference between the group A and B was detected. The highest value of velocity parameters was observed in NEA 532 (group A). Linearity and amplitude of lateral head displacement are parameters supportive in identification of hyperactivated spermatozoa. Because movement of the spermatozoa head depends on flagellar bend and beat patterns, increased VCL and ALH as well as decreased LIN are indicative of hyperactivation (**Mortimer *et al.*, 1990**). No significant differences were detected in amplitude of lateral head displacement between the group A and B as well as between individual bulls (Table 1). In the previous study **Farrel *et al.* (1998)** evaluated

spermatozoa parameters with a CASA and identified several parameters highly correlated with non-return rates in dairy cattle. The parameters found to be associated with bull fertility were: percent of progressive spermatozoa, path (VAP) and progressive (VSL) velocity, track speed (VCL), linearity and straightness of the spermatozoa path, lateral amplitude (ALH) of spermatozoa head movement, and spermatozoa tail beat frequency (BCF).

Table 1 Selected movement characteristics of frozen-thawed bull spermatozoa.

		VAP	VCL	VSL	LIN	ALH
Group A	NEA 737	63.74± 1.71	109.11±2.12	50.59±2.26	0.46	4.23±0.15
	BA100	65.80±1.77	124.96±3.56	50.70±1.81	0.40	4.47±0.20
	NEA 532	72.16±1.85	137.26±4.31	58.65±2.23	0.43	4.43±0.19
Group B	AMT 55	64.11±2.31	126.68±4.47	49.48±1.86	0.39	4.58±0.23
	RAD 229	63.95±1.13	129.54±2.01	52.36±1.44	0.40	4.12±0.15
	NAE 356	63.34±1.79	129.75±3.48	47.40±1.88	0.36	4.60±0.16

Legend: VCL – curvilinear velocity (μm.sec^{-1}) ; VAP – average path velocity (μm.sec^{-1}) ; VSL – straight line velocity (μm.sec^{-1}) ; ALH – amplitude of lateral head displacement (μm) ; LIN – linearity VSL / VCL (linearity of the curvilinear trajectory)

Spermatozoa freezing have also been reported to result in a reduction in spermatozoa viability, changes in spermatozoa function, lipid composition and organization of the spermatozoa plasma membrane (**Bailey *et al.*, 2000**). PNA is a routinely used lectin to label the acrosome part. PNA lectin has been shown to bind Galß (1–3) Gal NAC residues (**Varki *et al.*, 1999; Sharon, 2007**) located on the outer acrosomal membrane (**Mortimer *et al.*, 1987; Martinez-Menarguez *et al.*, 1992; Aviles *et al.*, 1997**). In unfixed spermatozoa samples PNA marked only spermatozoa with disturbed plasma membrane integrity. Proportion of PNA positive spermatozoa in all tested group of bulls ranged from 14.97% (group A) to 29.84% (group B). It was significantly lower ($P<0.001$) proportion of spermatozoa with damaged integrity of the membrane in the group A with better motility parameters compared to the group B (Fig 2).

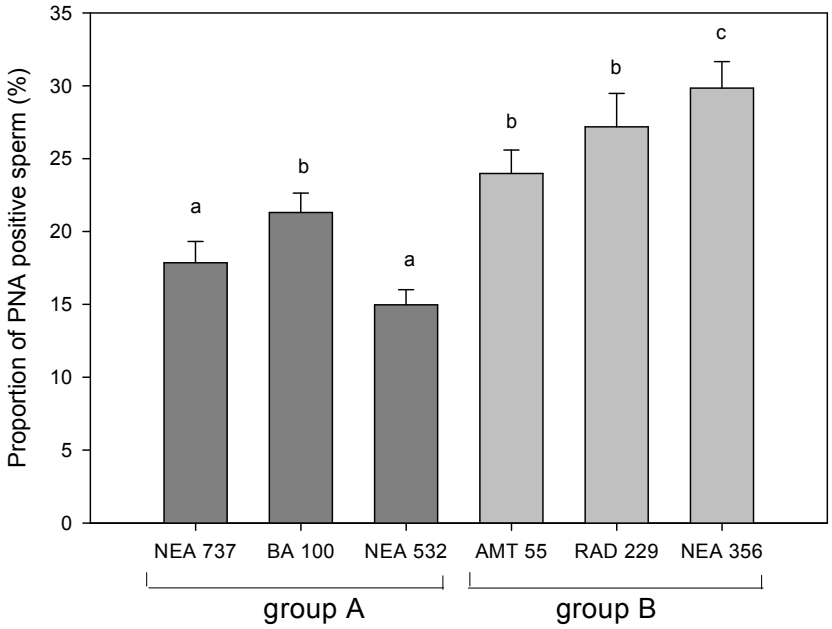

Figure 2 Proportion of spermatozoa with disturbed membrane integrity in frozen-thawed bull semen. a, b, c – differences are significant ($P < 0.001$) when compared the group A versus the group B

Similarly incidence of apoptotic cells (Fig 3) was significantly lower ($P<0.001$) in the group A (NEA 532, BA 100, NEA 737). Presence of apoptotic spermatozoa in the semen dose could be one of the reasons for poor fertility of breeding bulls (**Anzar *et al.*, 2002**) and similarly spermatozoa with damaged or inactive membranes will have limited viability and fertilization potential (**Correa and Zavos, 1994**).

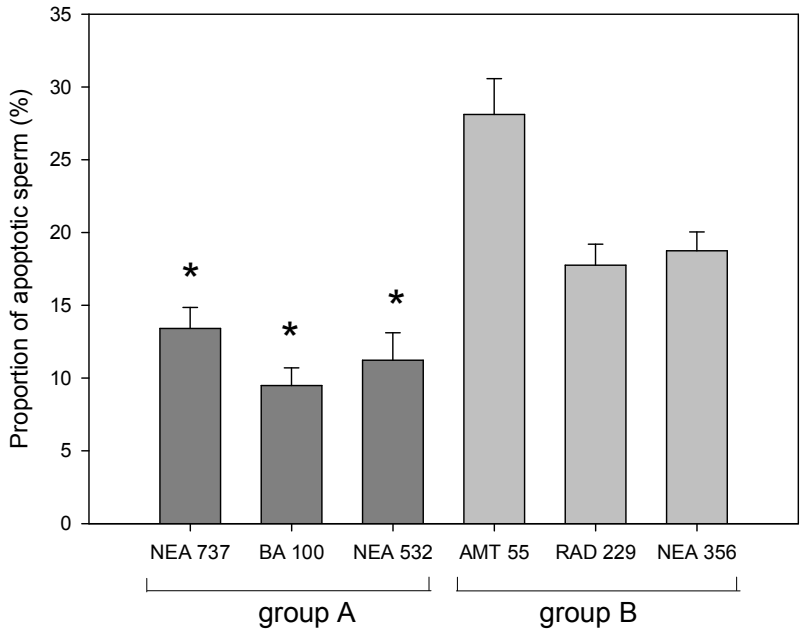

Figure 3 Proportion of apoptotic spermatozoa in frozen-thawed bull semen. * - significant difference between the group A and B at $P < 0.001$

Procedures necessary for cryopreservation of spermatozoa include dilution, freezing and thawing which are known to damage plasma membranes (**Hammerstedt *et al.*, 1990; Parks *et al.*, 1992**). It is generally accepted that at least 50% of spermatozoa die during the freezing and thawing procedures (**Watson, 2000**). In our work, proportion of dead/necrotic spermatozoa was significantly lower in the group A ($P<0.001$) (Fig 4).

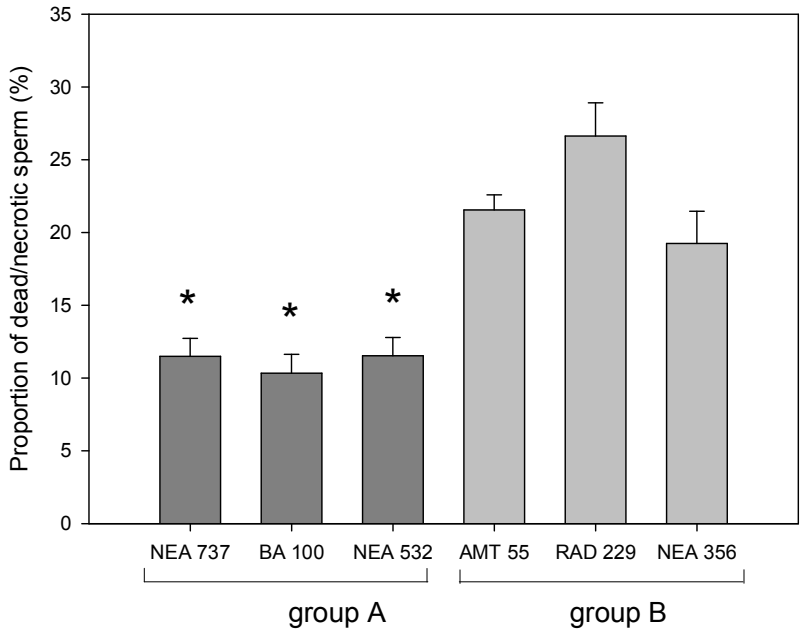

Figure 4 Proportion of dead/necrotic spermatozoa in frozen-thawed bull semen. * - significant difference between the group A and B at $P < 0.001$

Results of in vitro fertilization test are shown in Table 2. Impact of individual bull on cleavage rate of embryos at day 2 was observed. Bulls in the group A with better motility and viability parameters show significantly higher cleavage rate of the embryos.

Table 2 Cleavage rate of embryos in *in vitro* fertilization test

		Total no. oocytes	Cleavage embryos (D2), n	Cleavage rate (D2), %
Group A	NEA 737	179	139	77.65
	BA100	160	97	60.63
	NEA 532	120	86	71.67
Group B	AMT 55	38	10	26.32
	RAD 229	117	35	29.91
	NEA 356	75	18	24.00

Cryopreservation causes irreversible damage to spermatozoa organelles, and changes in membrane fluidity and enzymatic activity, associated with a reduction in spermatozoa motility, viability and fertilizing ability (**Alvarez and Storey 1989; Hammerstedt 1993**). Good post-thawing motility parameters in connection with low proportion of dead, apoptotic

spermatozoa and spermatozoa with damaged plasma membrane led to an improvement of fertilization ability and higher percentage of cleavage rate of embryos.

CONCLUSION

In conclusion, our results indicate close relationship between motility and viability parameters of frozen-thawed bull spermatozoa. Spermatozoa with higher total motility and progressive movement had better parameters of spermatozoa viability, except spermatozoa velocity parameters and amplitude of lateral head displacement. These motility and viability characteristics are associated with a higher embryo cleavage rate in in vitro fertilization test.

Acknowledgments: The study was supported from the grants NAZV QI91A061 (Czech Republic).

REFERENCES

AIRES, V.A. – HINSCH, K.D. – MUELLER-SCHLOESSER, F. – BOGNER, K. – MUELLER-SCHLOESSER, S. – HINSCH, E. 2003. In vitro and in vivo comparison of egg yolk-based and soybean lecithinbased extenders for cryopreservation of bovine semen. In *Theriogenology*, vol. 60, 2003, p. 269–279.

ALVAREZ, J.G. – STOREY, B.T. 1989. Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. In *Gamete Research*, vol. 23, 1898, p. 77-90

AMIRAT, L. – TAINTURIER, D. – JEANNEAU, L. – THORIN, C. – GERARD, O. – COURTENS, J.L. – ANTON, M. 2004. Bull semen in vitro fertility after cryopreservation using egg yolk LDL: a comparison with Optidyl®, a commercial egg yolk extender. In *Theriogenology*, vol. 61, 2004, p. 895–907.

ANZAR, M. – HE, L. – BUHR, M. M. – KROETSCH, T. G. – PAULS, K. P. 2002. Sperm apoptosis in fresh and cryopreserved bull semen detected by flow cytometry and its relationship with fertility. In *Biology of Reproduction*, vol. 66, 2002, p. 354-360.

AVILES, M. – CASTELLS, M.T. – MARTINEZ-MENARGUEZ, J.A. – ABASCAL, I. – BALLESTA, J. 1997. Localization of penultimate carbohydrate residues in zona pellucida and acrosomes by means of lectin cytochemistry and enzymatic treatments. In *Histochemistry*, vol. 29, 1997, p. 583–592.

BAILEY, J. L. – BILODEAU, J. F. – CORMIER, N. 2000. Semen cryopreservation in domestic animals: a damaging and capacitating phenomenon. In *Journal of Andrology*, vol. 21, 2000, p. 1–7.

CORREA, J. R. – ZAVOS, P. M. 1994. The hypoosmotic swelling test : Its employment as an assay to evaluate the functional integrity of the frozen-thawed bovine sperm membrane. In *Theriogenology*, vol. 42, 1994, p. 351-360.

FARRELL, P.B. – PRESICCE, G.A. – BROCKETT, C.C – FOOTE, R.H. 1998. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. In *Theriogenology,* vol. 49, 1998, p. 871-879.

GIL, J. – RODRIGUEZ-IRAZOQUI, M. – LUNDEHEIM, N. – SODERQUIST, L. – RODRIGUEZ-MARTINEZ, H. 2003. Fertility of ram semen frozen in Bioxcell and used for cervical artificial insemination. In *Theriogenology*, vol. 59, 2003, p. 1157-1170.

HAMMERSTEDT, R.H. 1993. Maintenance of bioenergetic balance in sperm and prevention of lipid peroxidation: a review of the effect on design of storage preservation systems. In *Journal of Reproduction and Fertility*, vol. 5, 1993, p. 675-690.

HAMMERSTEDT, R.H – GRAHAM, J.K. – NOLAN, J.P. 1990. Cryopreservation of mammalian sperm: what we ask them to survive. In *Journal of Andrology*, vol. 11, 1990, p. 73–88.

CHATTERJEE, S. – DE LAMIRANDE, E. – GAGNON, C. 2001. Cryopreservation alters membrane sulfhydryl status of bull spermatozoa: protection by oxidized glutathione. In *Molecular Reproduction and Development*, vol. 60, 2001, p. 498-506.

JEYENDRAN, R. – ACOSTA, V. – LAND, S. – COULAM, C. 2008. Cryopreservation of human sperm in a lecithin-supplemented freezing medium. In *Fertility and Sterility*, vol. 90, 2008, p. 1263-1265.

MARTINEZ-MENARGUEZ, J.A. – BALLESTA, J. – AVILES, M. – CASTELLS, M.T. – MADRID, J.F. 1992. Cytochemical characterization of glycoproteins in the developing acrosome of rats. In *Histochemistry*, vol. 97, 1992, p. 439–449.

MORTIMER, S.T – MORTIMER, D. 1990. Kinematics of human spermatozoa incubated under capacitating conditions. In *Journal of Andrology*, vol. 11, 1990, p. 195–203.

MORTIMER, D. – CURTIS, E.F. – MILLER, R.G. 1987. Specific labeling by peanut agglutinin of the outer acrosomal membrane of the human spermatozoon. In *Journal of Reproduction and Fertility*, vol. 81, 1987, p. 127–135.

PARKS, J.E. – GRAHAM, J.K. 1992. Effects of cryopreservation procedures on sperm membranes. In *Theriogenology*, vol. 38, 1992, p. 209–22.

PERSON, C.N – LESTER, M.D. – PERSON, M.D. – RORIE, R.W. 2007. Effects of penicillamine, hypotaurine and epinephrine (PHE) on post-thaw bovine sperm parameters, as measured by computer-assisted sperm analysis. *Arkansas Animal Science Department Report*, 2007, p. 47-50. http://arkansasagnews.uark.edu/553-11.pdf

SARIÖZKAN, S. – TUNCER, P.B. – BUCAK, M.N. – ULUTAŞ, P.A. 2009. Influence of various antioxidants on microscopic-oxidative stress indicators and fertilizing ability of frozen-thawed bull semen. In Acta Veterinaria Brno, vol. 78, 2009, p. 463-469.

SHARON, N. 2007. Lectins: carbohydrate-specific reagents and biological recognition molecules. In *Journal of Biological Chemistry.* Vol. 282, 2007, p. 2753–2764.

VARKI, A. – CUMMINGS, R. – ESKO, J. – FREEZE, H. – HART, G. – MARTH, J. 1999. Plant lectins. In: Van Damme, J.M. (Ed.), Essentials of Glycobiology. In *Cold Spring Harbor Laboratory Press*, New York, 1999, p. 455–467.

WATSON, P.F. 2000. The causes of reduced fertility with cryopreserved semen. In *Animal Reproduction Science*, vol. 60, 2000, p. 481–492.

WATSON, P.F. 1976. The protection of ram and bull spermatozoa by the low density lipoprotein fraction of egg yolk during storage at 5 °C and deep-freezing. In *Journal of Thermal Biology*, vol. 1, 1976, p. 137-141.

WHITE, I.G. 1993. Lipids and calcium uptake of sperm in relation to cold shock and preservation: A review. In *Reproduction*, *Fertility and Development*, vol. 5, 1993, p. 639-658.