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MOTILITY AND FERTILIZING ABILITY OF FROZEN-THAWED RAM SPERM FROM TWO SHEEP BREEDS

E. KUBOVIČOVÁ[1]*, A. V. MAKAREVICH[1], E. ŠPALEKOVÁ[2], Z. HEGEDUŠOVÁ[3]

[1]Animal Production Research Centre Nitra, Slovak Republic
[2]Slovak University of Agriculture in Nitra, Slovak Republic
[3]Research Institute for Cattle Breeding, Ltd., Czech Republic

ABSTRACT

The aim of the study was to evaluate motility parameters of Lacaune (Lc) and East-Friesian (EF) ram frozen-thawed spermatozoa by CASA (Computer-Automated Sperm Analysis) technique in relation to their in vivo fertilizing ability. Fresh ejaculates were diluted in a Triladyl extender containing 20 % egg yolk, 6 % glycerol and 0.1 % trehalose and frozen in a liquid nitrogen vapour. The evaluation of frozen-thawed semen showed significant differences in sperm motility between the two sheep breeds by 30 ($P<0.001$) to 120 ($P<0.05$) min post thawing. Whole motility of spermatozoa was significantly reduced during incubation and by 120 min it was dropped to 7.68 (Lc) and 13.36 (EF) %. Similarly, the percentage of progressive motile spermatozoa was significantly reduced ($P<0.001$) during incubation, and only 6.31 % of Lc and 11.81 % of EF ram spermatozoa were progressive motile by 120 min of incubation. Significant decline of the velocity characteristics, such as a path velocity (VAP), curveilance velocity (VCL) and progressive velocity (VSL) was observed by 30 min ($P<0.001$) and 120 min ($P<0.05$) post thawing in Lc compared to the EF ram semen. Ram frozen-thawed semen was used for insemination of 99 hormonally synchronized ewes. The differences detected in the motility parameters of frozen-thawed spermatozoa from two sheep breeds were reflected in the pregnancy (12.24 and 28.00 %) and lambing rate (0.0 and 56.00 %) for EF and Lc, respectively.
In conclusion, cryopreservation significantly affects the quality of frozen-thawed sperm by lowering sperm motion characteristics. This study revealed differences in basal motility parameters between ram sperm of two sheep breeds.

Key words: ram; sperm; cryopreservation; CASA; motility; artificial insemination

INTRODUCTION

Artificial insemination (AI) with frozen semen has been proved as the most potent method for rapid genetic improvement in domestic animals. Although the basic principle of cryopreservation is similar for spermatozoa of most mammalian species, the sperm from different species may response to freezing differently, due to their difference in morphology and certain biochemical constituents. At each stage of the cryopreservation cycle, which includes the entire process of semen collection, dilution, equilibration and freezing, the spermatozoa may lose the ability to fertilize normally (Watson, 1995).

The maintenance of motility and other sperm characteristics during post-thawing incubation is an indicator of the usability of the semen (Saacke and White, 1972), which reflects a greater likehood of their survival in the female reproductive tract to undergo capacitation and fertilize ova (Fiser *et al*., 1993).

Ram spermatozoa are sensitive to extreme temperature changes during freezing processes (Salamon and Maxwell, 1995) leading to alterations of speramatozoa membrane integrity and ultrastructure (Watson, 1995). Quality tests are routinely used to determine acceptability of processed semen for breeding purposes. Sperm motility in general and characteristics of sperm motion in particular

***Correspondence:** E-mail: kubovicova@cvzv.sk
Elena Kubovičová, Animal Production Research Centre Nitra,
Hlohovecká 2, 951 41 Lužianky, Slovak Republic
Tel.: +421 37 6546 236 Fax: +421 37 6546 189

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could be some of the indicators of the spermatozoa quality. The principal laboratory tests for standard semen analysis use light microscopy to estimate sperm survival and the percentage of motile and progressively motile spermatozoa (Correa *et al*., 1997; Rodriguez-Martinez, 2003, Yamashiro *et al*., 2011). Although useful, these tests are not completely reliable or repeatable because of the small numbers of sperm evaluated, lack of objectivity and human bias (Graham, 2008). More objectivity and repeatability in assesing sperm motility can be achieved by computer automated sperm analysis - CASA (Davis and Siemers, 1995; Kumar *et al*., 2010). Besides the percentage of a whole motility, progressive and local motile sperm, using CASA it is possible to measure different parameters of velocity (for example velocity straight line - VSL, velocity curvilinear - VCL) or the beat cross frequency, BCF. The development of CASA and a variety of fluorescence dyes offer new possibilities, to achieve more and more objective information about the functionality of fresh and frozen/thawed sperm (Januskauskas *et al.*, 2003; Tardif *et al*., 1997).

Therefore, the aim of the study was to evaluate motility parameters of Lacaune and East-Friesian ram post-thawed spermatozoa by CASA in relation to *in vivo* fertilizing ability of spermatozoa.

MATERIAL AND METHODS

Semen collection, dilution and freezing

Fresh ejaculates were collected from three rams of Lacaune (Lc) and one of East-Friesian (EF) sheep breeds with proven health and reproductive status using an artificial vagina. The rams were kept at a local farm (Trenčianska Teplá, Slovak Republic) under uniform nutritional conditions. The ejaculates from Lc rams were pooled together to make heterospermia in order to avoid individual influence of the ram and were used for the assay. After measuring the volume, the density and activity, the ejaculates were diluted (1:3) in a Triladyl extender (Minitub Slovakia Ltd, Čeľadice, Slovak Republic) containing 20 % egg yolk, 6 % glycerol and 0.1 % trehalose, filled to the 0.1 ml straws and equilibrated in a refrigerator at 5°C for 1.5 hour. Then the straws were frozen in a liquid nitrogen vapour by suspending them horizontally 5 mm apart and 4 cm above a liquid nitrogen in a Styrofoam box (Minitube, SRN) for 20 minutes before plunging them into a liquid nitrogen.

Assessment of post-thawing characteristics of spermatozoa

The frozen semen from the Lc and EF rams was thawed by placing individual straws into the water at 70ºC for 8 s. Sperm motility parameters were measured using CASA system (Sperm Vision, Minitüb Slovakia Ltd., Čeľadice, Slovak Republic) at 30, 60 and 120 min post thawing. Spermatozoa were transferred by a pipete into a Laja counting chamber with the depth of 10 µm. The chamber was placed under a Zeiss Axioscope A.1 phase-contrast microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) with heating stage (37°C) at 200 x magnification. The image was transfered into a computer by the camera; sperm motility was measured by the Sperm Vision software. At least 6 view fields per each sample were counted. The whole motility (M), progressive motility (PM) and the velosity characteristics, namely path velocity (VAP), curveilance velocity (VCL) and progressive velocity (VSL) of the sperm were evaluated in this study. The values of these parameters measured at several time points (30, 60 and 120 min) were summarized and the everage values were presented in the table and graphs.

Synchronization of estrus and artificial insemination

A total of 99 Lacaune (LC), Tsigai (TS) and Improved Valachian (IV) mature ewes (2-3 years old) were used for insemination as described earlier (Kubovičová *et al*., 2010). Briefly, the ewes were treated with 40 mg fluoroprogesterone acetate (Cronolonum; Intervet International, Boxmeer, Holland) using an intravaginal sponge, inserted into a vagina of ewes for 14 days. On the day of sponge removal, 500 IU of pregnant mare serum gonadotropin (Werfaser, Werff Chemie; Austria) were injected intramuscularly. Following 48-57 hours after sponge removal the ewes were cervically inseminated. The semen dose was thawed at 70°C for 8 s and an insemination dose of approximately 100×10^6 spermatozoa was deposited as far as possible into the first fold of the cervix. After 24 hours the ewes were re-inseminated. Pregnancy was diagnosed 60 days after AI by a real-time ultrasonic scan device (Alloka SSD 500).

Statistical analysis

The results were statistically evaluated by two-way ANOVA test and graphically processed using SigmaPlot graphic software (version 9.01 for Windows).

RESULTS AND DISCUSSION

In the present study there were no significant differences in a whole sperm motility (M), progressive motility (PM) and velosity characteristics between the two groups of ram semen immediately after thawing (0 min) (Table 1).

Table 1: Motility parameters of ram frozen-thawed spermatozoa evaluated by CASA

Parameters	0 min		30 min		120 min	
	Lacaune	East-Friesian	Lacaune	East-Friesian	Lacaune	East-Friesian
M (%)	28.95±0.90	22.19±1.43	17.10±1.61[a]	22.17±1.49[b]	7.68 ± 0.47[c]	13.36±0.90[d]
PM (%)	24.44±0.79	19.60±1.60	11.54±0.78[a]	18.30±1.22[b]	6.31 ±0.43[c]	11.81±1.17[d]
Total Cells (n)	338	603	240	593	340	326
DAP	38.39±2.39	39.58±2.87	29.33±3.35	44.61±1.77	34.13±2.33	37.53±4.45
DCL	82.72±7.37	78.22±5.06	57.56±8.51	78.68±3.23	51.46±3.58	58.32±7.55
DSL	30.39±1.13	32,98±2.94	25.14±3.22	41.04±1.31	30.95±2.81	34.54±4.28
VAP (μm sec^{-1})	85.99±4.65	92.01±6.08	63.26±6.56[a]	105.35±4.63[b]	74.21±4.48[c]	86.49±9.85[d]
VCL (μm sec^{-1})	185.09±15.00	181.78±11.22	123.13±17.29[a]	185.12±7.80[b]	112.29±7.79[c]	133.52±16.86[d]
VSL (μm sec^{-1})	68.25±2.53	76.74±6.08	53.81±6.23[a]	97.14±3.54[b]	67.34±5.37[c]	79.24±9.44[d]
STR (%)	0.79±0.02	0.83±0.01	0.84±0.03	0.92±0.01	0.90±0.02	0.91±0.01
LIN (%)	0.37±0.02	0.42±0.01	0.46±0.05	0.52±0.01	0.60±0.04	0.60±0.03
WOB	0.46±0.01	0.05±0.01	0.54±0.04	0.57±0.01	0.67±0.03	0.66±0.03
ALH (μm)	5.81±0.28	5.80±0.16	3.35±0.30	4.63±0.23	2.71±0.38	3.80±0.39
BCF (Hz)	27.28±1.42	31.35±1.29	29.44±4.05	37.40±0.41	38.68±2.79	34.66±1.16

[b,a] $P<0.001$, [d,c] $P<0.05$

Whole motility of spermatozoa was significantly reduced during an incubation, and by 120 min it was dropped to 7.68 (Lc) and 13.36 (EF) % (Fig. 1A). Similarly, the percentage of progressive motile spermatozoa was significantly reduced ($P<0.001$) during incubation, and only 6.31 % of Lc and 11.81 % of EF ram spermatozoa were progressive motile by 120 min of incubation (Fig. 1B).

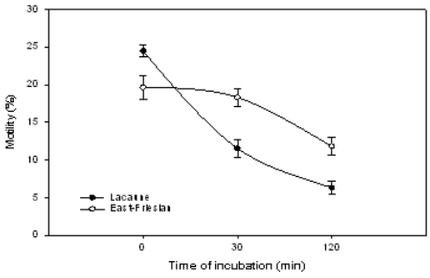
Maintenance of sperm function during freezing and thawing depends upon several interrelated factors which include cooling rate, equilibration period and freezing technique (Salamon and Maxwell, 2000; Bailey *et al*., 2000; Curry, 2000; Anel *et al*., 2006) but their adverse effects are manifested on thawing (Holt and North, 1994). The degree of cryo-damages also depends on several factors (Watson, 2000; Naqvi *et al*., 2001), which limit the survival of spermatozoa during incubation (Aisen *et al*., 2000; Bagg *et al*., 2002). Thus, detailed semen examination is an important aspect that must be accurately done to ensure the use of breeding males with a good fertility. The CASA technique can provide rapid, precise and validated objective measurements of the spermatozoa motion characteristics (Holt and Palomo, 1996). Centola (1996) demonstrated that CASA gives much more detailed results that are less changed by errors than the manual microscopic observation. Motility is one of the parameters most seriously affected by freezing (Watson, 1995).

Relationship between motility characteristics of human, bovine or porcine spermatozoa measured by computer–assisted instruments and their fertility in vitro, in vivo or fertility indexes have been reported by several researchers (Aitken, 1989; Vestergen *et al.*, 2002). Farrel et al. (1998) found a high correlation between several motility characteristics and 59-day non-return to estrus rates of cows inseminated with frozen-thawed spermatozoa.

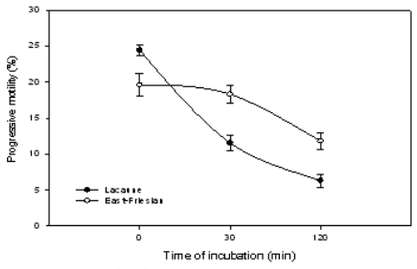
The measurement of sperm velosity has been considered as an indirect indicator of mitochondrial function of spermatozoa (Graham *et al*., 1984) and is associated with fertility (Budworth *et al*., 1988). The ram sperm motility characteristics are declined with the incubation time (Bag *et al*., 2002). In our study, a highly significant decline in the motility, progressive motility and the velosity characteristics, namely path velocity (VAP; Fig. 1C), curveilance velocity (VCL; Fig. 1D), progressive velocity (VSL; Fig. 1E) was observed by 30 min ($P<0.001$) and significant ($P<0.05$) by 120 min after thawing in the Lacaune, compared to the East-Friesian ram sperm ejaculate. One possible reason for this decline may be an inability of the spermatozoa to generate ATP through mitochondrial respiration as a consequence of mitochondrial aging or toxic effect of dead sperm associated with the liberation of amino oxidase activity (Cummins *et al.*, 1994; Visvanath and Shannon, 1997).

Maxwell and Watson (1996) also reported the toxicity of cryoprotective agent glycerol in semen diluents to bring about a reduction in sperm motility and to alter the acrosome integrity by interfering with the permeability of the sperm membrane. The choice of cryoprotectants

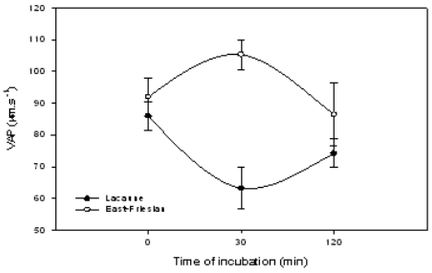
Fig. 1 A-F: Parameters of motility of ram frozen-thawed sperm from two sheep breeds



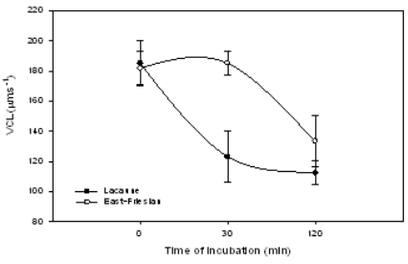
A: Whole motility



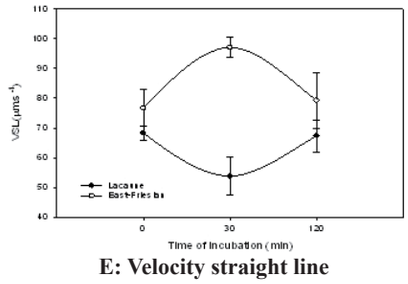
B: Progressive motility



C: Velocity average path



D: Velocity curved line



E: Velocity straight line

is based not only on their ability to protect sperm during freezing, but also on their molecular weight, which is important in reducing the high osmolarity-induced sperm toxicity by faster and easier cellular penetration (Artifiantiny *et al*., 2010).

In the field trial, the differences which we found out in the motility parameters of frozen-thawed semen from two ram breeds, were reflected in the pregnancy (12.24 and 28.00 respectively; $P<0.001$) and lambing rate (0.0 and 56.00 respectively; $P<0.001$) of ewes inseminated cervically with frozen-thawed semen collected from East-Friesian and Lacaune rams.

A possible explanation of poorer quality of the Lacaune ram semen after thawing may be also a variation between the breeds. The effect of breed on sperm motion chracteristics was reported by Kumar *et al*. (2010), who compared Malpura and Barat Merino rams. The breed had significant ($p<0.05$) effect on VCL,VAP, VSL, ALH, BF, sperm head area, rapid motile sperm, medium motile sperm, slow motile sperm, LIN, STR and sperm head elongation, with higher values of almost all the sperm motion characteristics in the Malpura breed.

Male-to-male differences in freezing and thawing tolerance of sperm have been reported for several species (Thurston *et al*. 2001, Allessandro and Martemucci, 2003). Leboeuf *et al*. (2000) and Medrano *et al*. (2010) showed in goat semen that such variability is relatively independent of prior semen quality and the semen of certain individuals consistently freezes with less cryoinjury than that of others. The fact that males can often be classified as good freezers or bad freezers implies that certain characteristics of membrane structure, which may be genetically determined, predispose towards survival under cryopreservation stress (Watson, 2000). There are instances, where certain semen samples within a species, having good pre-freeze motility, still results in a poor freezability.

Recent study (Thurston *et al*., 2002) suggests that there is also a genetic basis for variation in post-thaw semen quality, and argue that modern molecular technologies are able to identify markers linked to genes influencing this variation. On the basis of identification of differences in post-thaw survival among individual rams it will be possible to select proper rams, whose sperm will be suitable for cryopreservation.

CONCLUSION

In conclusion, cryopreservation significantly affects the quality of frozen-thawed semen by lowering sperm motion characteristics. This study revealed differences in basic motility parameters between ram sperm of two sheep breeds.

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