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Motility Parameters and Intracellular ATP Content of Rabbit Spermatozoa Stored for 3 Days at 15°C

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The effect of semen storage duration on motility parameters and ATP content of rabbit spermatozoa were investigated. Ejaculates were collected from 9 New Zealand White male rabbits and diluted with a commercial rabbit semen extender Galap. Semen was stored at 15°C for 3 days. On each day of storage sperm motility and intracellular ATP content were evaluated. Sperm motility parameters were assessed using the computer-assisted sperm analysis (CASA) system and ATP content using the bioluminescence method. The time of storage had a significant effect on sperm motility parameters (except straight-line velocity) and ATP content. A significant correlation was observed between motility parameters and sperm ATP content. The motility parameters most strongly correlated with ATP content were total motile spermatozoa (r = 0.6364), progressively motile spermatozoa (r = 0.529), amplitude of lateral head displacement (r = 0.4178), curvilinear velocity (r = 0.4111) and average path velocity (r = 0.3743). Results show that motility parameters determined using the CASA system and intracellular ATP content are sensitive indicators of sperm quality during *in vitro* storage and may be useful for estimation of *in vivo* fertilizing ability of rabbit semen.

Key words: Rabbit, semen storage, sperm motility, CASA, ATP content.

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As an important part of reproductive technology, insemination is an increasingly used method in rabbit breeding. Today, both fresh semen and short-term liquid preserved semen are - almost exclusively – used in artificial insemination practice. Frozen semen has lower biological value, which generally translates into lower fertility and prolificacy after use (GOGOL 1997; MOCE *et al.* 2010). This fact and the need for considerable selection of males and ejaculates in terms of the suitability of semen for freezing is the main factor limiting the widespread application of semen from outstanding sires. As a consequence, efforts are made to extend semen storage time above zero temperatures without reducing fertility parameters (ROCA *et al.* 2000; LOPEZ-GATIUS *et al.* 2005). The possibility of semen storage for 2-3 days, while maintaining the fertilizing capacity of spermatozoa, would facilitate the transport and extensive use of genetically superior buck semen for artificial insemination in rabbits.

The effectiveness of the fertilizing process depends primarily on the *in vivo* activities of sperm cells and on the efficiency of their complex axoneme, which ensures the normal transport of genetic material carried by sperm. It is therefore important to observe and evaluate them. This evaluation can be done in two ways: by determining the motility of sperm and by determining the intensity of biochemical processes characterizing sperm cell metabolism. Evaluation of the motility of sperm is the basic and most common test. It is generally believed that motility and fertility are related, although data from the literature are inconclusive and the coefficients of correlation obtained are low (GADEA 2005). The few preliminary studies with rabbit semen indicate however that the computer-assisted sperm analysis (CASA) system can be used to predict the fertilizing capacity of spermatozoa (LAVARA *et al.* 2005; QUINTERO-MORENO *et al.* 2007). Only one study has evaluated the impact of long-term storage on CASA motility parameters of rabbit spermatozoa in solidified extender (LOPEZ-GATIUS *et al.* 2005).

Energy metabolism is a key factor needed for normal sperm function and maintenance of sperm motility. ATP is generated by oxidative phosphorylation in mitochondria or by substrate phosphorylation during glycolysis. ATP, which carries

chemical energy in cells, is essential primarily for sperm motility together with contractile proteins present in the flagellum as well as for the generation of membrane potentials by ATPases. Measurement of ATP level may provide information on the efficiency of metabolic mechanisms that control energy production and consumption in the cell. However, data concerning the relationship between ATP level, sperm motility and fertility are inconsistent (GUMIŃSKA *et al.* 1997; MINELLI *et al.* 1999; GOGOL *et al.* 2009; TORRES-FLORES *et al.* 2011) and this issue needs further study.

The aim of the study was to investigate the effect of long-term storage of rabbit semen on sperm motility parameters evaluated using the CASA system and to determine their correlation with sperm ATP content.

Material and Methods

Animals and experimental procedure

Semen of 9 sexually mature New Zealand White male rabbits was used in the experiment. Males were housed at the local experimental farm of the Department of Biotechnology of Animal Reproduction, National Research Institute of Animal Production (Balice, Poland). Animals were housed under a photoperiod of 14h light: 10h dark, in individual cages, fed with a commercial diet and provided water *ad libitum*. Temperature in the building was kept between 16-19°C. Semen was collected by means of an artificial vagina at weekly intervals for 5 weeks. Just after collection, ejaculate volume was determined and sperm concentration calculated using the CASA system. Ejaculates with at least 0.6 ml volume and sperm concentration in excess of $200x10^6$/ml were qualified for the study. Each qualified ejaculate (2 to 4 ejaculates per male) was diluted at a 1:10 ratio with rabbit semen extender Galap (IMV, France) and stored for 3 days at 15ºC.

On each day of semen storage a 0.5 ml aliquot of each sample was removed and warmed to 37°C for 30 minutes in a water bath. The sample was then analyzed for characteristics of sperm motility and ATP content.

Assessment of sperm motility

Sperm motility parameters were assessed using a computer-assisted sperm analysis (CASA) system (Sperm Class Analyzer, S.C.A V5.1, Microptic, Barcelona, Spain). The CASA analysis was based on the following set-up: capture of 25 consecutive frames per second, image acquisition time was 1 second per field, minimum number of consecutive images per spermatozoa was 10, the particles captured were between 10-80 μm^2. Spermatozoa were considered motile when VCL $\geq$ 10 μm/s and progressive when STR was at least 70%. Immediately after gentle mixing a total of 3 μl of sample was placed in a prewarmed Leja standard count 4 chamber slide (Leja Products B.V., The Netherlands). Sperm motility was assessed with a microscope equipped with a 10x negative-phase contrast objective and a heated stage at 37°C. For each sample a minimum of five microscopic fields were analyzed and a minimum of 500 sperm evaluated. The following characteristics of sperm motility were determined: total motile spermatozoa (TMOT, %), progressively motile spermatozoa (PMOT, %), curvilinear velocity (VCL, μm/s; the average velocity measured over the actual point to point track followed by the cell), straight-line velocity (VSL, μm/s; the average velocity measured in a straight line from the beginning to the end of the track), average path velocity (VAP, μm/s; the average velocity of the smoothed cell path), linearity (LIN, %; the average value of the ratio VSL/VCL), straightness (STR, %; the average value of the ratio VSL/VAP), amplitude of lateral head displacement (ALH, μm; the mean width of the head oscillation as the sperm cells swim), and beat cross-frequency (BCF, Hz; the frequency of sperm head crossing the average path in either direction).

Adenosine triphosphate measurement

The adenosine triphosphate (ATP) from spermatozoa cells was determined using the ViaLight Plus kit according to the manufacturer's instructions (Cambrex Bio Science Rockland, Inc., USA). The kit is based upon the bioluminescent measurement of ATP in mammalian cells. The bioluminescent method utilizes the enzyme luciferase, which catalyses the production of light from ATP and luciferin. The emitted light intensity is linearly related to the ATP concentration and is measured using a luminometer. Prior to assay, samples composed of 10 μl of diluted semen were mixed with 100 μl Cell Lysis Reagent and incubated at room temperature for 5 minutes to extract ATP from cells. Following the addition of 100 μL ATP Monitoring Reagent via automated dispensers, luminescence was measured using an AutoLumat LB953 luminometer (Berthold, Bad Wildbad, Germany). The signal generated was compared to standard ATP dilutions. The reaction mixture used for standard curves consisted of a 10 μl ATP dilution, 100 μl of Cell Lysis Reagent and 100 μl ATP Monitoring Reagent. The standard curve covered the range from 0 to 100 pM ATP in the reaction mixture. Sperm ATP content from each probe was assessed in duplicate.

Table 1

Effect of storage time on parameters of sperm motility and intracellular ATP content (Mean ± SE; n=26)

Parameter	Day 0	Day 1	Day 2	Day 3
TMOT (%)	68.8±3.0a	62.0±2.2ab	51.2±2.2b	36.2±2.8c
PMOT (%)	56.0±3.1a	49.7±2.3ab	39.1±2.1b	26.7±2.7c
VCL (μm/s)	96.6±5.1a	89.2±4.0ab	76.7±4.0bc	71.5±4.4c
VSL (μm/s)	40.6±2.9	46.5±3.6	39.3±3.6	40.3±3.9
VAP (μm/s)	71.1±4.9a	66.7±4.1ab	54.5±4.2b	52.9±4.4b
LIN (%)	41.3±1.8a	50.9±2.2ab	49.8±2.8ab	53.5±2.5b
STR (%)	56.9±1.7a	68.5±1.6b	70.6±1.8b	73.6±1.5b
ALH (μm)	3.2±0.1a	2.8±0.1b	2.7±0.1bc	2.5±0.1c
BCF (Hz)	6.8±0.2a	7.9±0.2b	8.3±0.2b	8.0±0.2b
ATP (pmol/10^6sperm)	97.0±8.1a	86.5±6.4ab	67.7±4.5b	66.6±4.1b

SE: standard error
Values in rows with different letters are significantly different: a, b, c ($P<0.05$).

Statistical analysis

Data were subjected to variance analysis according to the GLM procedure of the Statistical Analysis System (SAS, version 8.2). The fixed effect of the day of storage was analyzed. The significance of differences between means was tested by Tukey's studentized range test. The values of all sperm parameters were expressed as mean ± SE. The correlations between sperm quality parameters were calculated using Spearman's rank method.

Results

The duration of storage had a significant effect on the motility parameters and the ATP content in sperm cells (Table 1). Effect of storage duration was significant for the percentages of total and progressively motile spermatozoa, VCL, LIN, STR, ALH, BCF ($P<0.01$) as well as VAP ($P<0.05$); however, there was no effect on the VSL parameter ($P>0.05$). The content of ATP decreased from 97.0 to 66.6 pmol/10^6 spermatozoa from day 0 to day 3 of storage ($P<0.01$).

Significant correlations were observed between motility parameters and ATP concentration (Table 2). The motility parameters most strongly correlated to ATP concentration were TMOT (r = 0.6364), PMOT (r = 0.529), ALH (r = 0.4178), VCL (r = 0.4111) and VAP (r = 0.3743). LIN was the only parameter to show no correlation to ATP content.

Table 2

Correlations between motility parameters and ATP content

Motility parameter	ATP
TMOT	0.6364 ($P<0.0001$)
PMOT	0.5290 ($P<0.0001$)
VCL	0.4111 ($P<0.0001$)
VSL	0.2352 ($P = 0.0162$)
VAP	0.3743 ($P<0.0001$)
LIN	-0.0235 ($P = 0.8127$)
STR	-0.2124 ($P = 0.0304$)
ALH	0.4178 ($P <0.0001$)
BCF	-0.0803 ($P = 0.0416$)

Based on means of 104 semen samples evaluated.
$P<0.05$ was considered significant.

Discussion

In the present study a significant effect of semen storage duration on CASA motility parameters was observed. Total and progressive motility were the parameters of rabbit sperm movement most strongly affected during semen storage (a decrease by 32.6 and 29.3%, respectively). In addition, these parameters were most strongly correlated to sperm ATP content. Total motility was the most significant parameter in prediction of rabbit fertility since a correlation was obtained between the

percentage of total motile sperm cells in fresh rabbit semen and the percentage of fertilized oocytes 42 h after insemination (HAGEN *et al.* 2003) and the kindling rate (LAVARA *et al.* 2005).

Of the velocity parameters examined in this study, the VCL value decreased the most during semen storage showing that this parameter is highly useful for evaluating the quality change of preserved rabbit semen.

LAVARA *et al.* (2005) reported that regression models including motility and morphological parameters of rabbit spermatozoa explained 45% of the variation in kindling rate. Parameters with the highest impact in this model were the velocity parameters VAP, VCL and the LIN. Two of them (VCL and LIN) have been associated with the hyperactivity motility pattern of the sperm (SCHMIDT & KAMP 2004). Since hyperactivation is a motility pattern observed in spermatozoa undergoing capacitation, samples showing this motility pattern may remain viable for a shorter time and therefore have a low *in vivo* fertilizing ability. For this reason, the identification of hyperactivated sperm is also important. Hyperactive mammalian spermatozoa have been characterized by a vigorous and non-linear movement caused by increased amplitude of flagellar beats (YANAGIMACHI 1994). The changes in parameters related to sperm hyperactivation observed in our study, i.e. increased LIN, decreased ALH and VCL as well as no significant changes in VSL, suggest that this phenomenon did not occur during the 3 days of storage at 15°C of rabbit semen diluted with Galap extender.

Results of the current study and previous research (ROCA *et al.* 2000; GOGOL & BOCHENEK 2003; GOGOL & WIERZCHOŚ-HILCZER 2009) show that a decreased percentage of motile spermatozoa and changes in motility characteristics observed during semen storage could result from damage to the axoneme with only moderate loss of integrity of cell membranes. Damage like this may occur due to the action of free radicals under oxidative stress (GUTHRIE & WELCH 2012). CASTELLINI *et al.* (2000) found that peroxidation is one of the main causes of rabbit sperm deterioration during conservation. The attack of free radicals on the unsaturated fatty acid-rich lipids of sperm cell membranes leads to irreversible reduction of membrane fluidity and to damage of cell membrane related ATPases, which are responsible for regulation of the intracellular level of ions necessary to maintain normal sperm motility (RAO *et al.* 1989). It is interesting that ATP production remained almost constant in days 2 and 3, although a marked decrease in motility was observed (from 51.2% to 36.2%). Perhaps in some situations immotile sperm can still produce ATP but the supply of ATP to the dynein ATPase, required for sperm motility, is limited. This phenomenon is not clear and needs further investigation.

The significant correlation between ATP concentration and several sperm motility parameters (especially TMOT, PMOT, ALH and VCL) indicates that ATP assessment may be useful as an additional, objective laboratory test. The results show that motility parameters determined using the CASA system and sperm ATP content are sensitive indicators of sperm quality changes during *in vitro* storage and may be useful in estimating the *in vivo* fertilizing ability of rabbit semen.

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