

ENZYME ACTIVITIES AND MOTILITY OF BOAR SPERMATOZOA DURING 72-HOUR LOW- TEMPERATURE STORAGE

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Summary

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The aim of this study was to determine the relationship between semen lactate dehydrogenase isoenzyme X (LDH-X) and gamma-glutamyl transferase (GGT) enzyme activities and sperm motility in boar ejaculates, stored at 15 °C for 72 h. Ejaculates were obtained from clinically healthy boars from Danube White breed. The motility, concentration and speed parameters of the sperms were determined by sperm class analyzer SCA (Microptic, Barcelona, Spain). The measurement of the spermatozoa enzyme activity was made by using a semi-auto clinical chemistry analyzer BA-88 (Mindray, Medical Germany GmbH, Bensheim, Germany). It was found that during storage, the decrease in the spermatozoa with progressive motility corresponded to reduced LDH-X and GGT enzyme activities. A stronger correlation was established between LDH-X activity and spermatozoa motility. In conclusion, LDH-X and GGT could be used as biological markers of semen quality after low-temperature (15 °C) storage for seventy two hours.

Key words: boar semen, gamma-glutamyl transferase (GGT), lactate dehydrogenase isoenzyme X (LDH-X), motility

INTRODUCTION

Utilisation of preserved semen for artificial insemination in pigs has increased approximately threefold in the past 15 years. More than 99% of inseminations per year in the world are made with semen that has been obtained on the same day, or stored at 15 to 20 °C for 1 to 5 days (Johnson *et al.*, 2000). The reduction of motility, which occurs during the storage has long been the main parameter used to evaluate the decrease in fertilising ability. Although inseminating spermatozoa are brought to the fertilisation site (the ovi-

duct) mainly by uterine contractions, high motility is required for the sperm to reach and penetrate the oocyte (Langendijk *et al.*, 2002).

During the storage, reorganisation of the sperm membrane lipids occurs leading to destabilisation in the cells that may cause changes in the sperm enzyme levels. These changes could therefore affect the sperm function and motility (Petrunkina *et al.*, 2007). Therefore, estimations of these enzymes have been recommended as markers for semen quality since they indi-

cate sperm damage (Singh *et al.*, 1996; Pesch *et al.*, 2006). For example, lactate dehydrogenase isoenzyme X (LDH-X) is an enzyme, specific for the reproductive tissue and is included in metabolic processes, which provide energy for survival, motility and fertility of spermatozoa (Blanco & Zinkham, 1963).

Gamma-glutamyl transferase (GGT) is another essential enzyme, important for the spermatozoa. Its function is associated with the glutathione metabolism and protection of spermatozoa from the oxidative stress (Sikka, 1996).

The aging-related functional changes within different compartments of the spermatozoa, such as the mitochondria, flagellum, plasma membrane and acrosome, are poorly understood. Therefore, a link between sperm motility and different enzymes levels (as a result of morphological changes) will provide a clearer picture of the semen storage effect.

The aim of this study was to determine the relationship between lactate dehydrogenase isoenzyme X (LDH-X), gamma-glutamyl transferase (GGT) enzyme activity and sperm motility in boar ejaculates, stored at a temperature as lower as 15 °C for seventy two hours.

MATERIALS AND METHODS

Animals

The experiment was conducted with four clinically healthy 3-year-old boars from the Danube White breed. They were housed on a private pig farm (Stomar-Invest, Belozem, Bulgaria). During the experiment, the boars were kept in boxes and were fed daily a balanced diet.

Control of the welfare and health status of the animals was performed on a daily basis.

Sperm collection and semen quality tests

The semen collection was performed through the artificial vagina method by an experienced operator in accordance with sex regimen of the boars. Initially, macroscopic evaluation of all eight ejaculates was performed and those out of standard requirements were discarded. Immediately after that, each ejaculate was diluted in a ratio 1:3 with semen diluents (Sredetz, Multidesign, Bulgaria), separated into four aliquots and carried to the lab for additional investigations. The volume (100 mL) and pH (7.5) of all samples were equalised. The first aliquots of each ejaculate were used for the analyses at 4th hour after semen collection and storage, while the other three were kept in an incubator at 15 °C for 24, 48, 72 hours, respectively.

All samples were submitted to routine semen assessment by sperm class analyser (SCA, Microptic S.L., Barcelona, Spain) to determine the sperm concentration ($\times 10^6/\text{mL}$), total sperm motility (%) and speed parameters (%) of spermatozoa.

Biochemical analyses

The profiles of LDH-X and GGT were determined in water and triton-soluble extract from spermatozoa at hours 24, 48 and 72.

After dilution the seminal plasma was removed by centrifugation at $3,000 \times g$ at 4 °C for 30 min. The pellet was washed and centrifuged 3 times at $3,000 \times g$ for 10 min at 4 °C with 0.9% saline, resuspended in distilled water and frozen for 12 h at -20 °C. Then the pellet was thawed, sonicated (150 W MSE ultrasonic disintegrator) and centrifuged at $12,000 \times g$ for 30 min at 4 °C. The obtained supernatant was used for analyses of water-soluble protein sperm fraction.

For obtaining triton extract, the pellet was resuspended with 1% Triton X-100 and centrifuged at the same conditions. Supernatants were used for analyses of membrane-bound protein fraction of spermatozoa.

The supernatants were used for determination of LDH-X and GGT activities. Measurements of their levels in the two extracts were made by commercial kit for semi-auto clinical chemistry analyzer BA-88 (Mindray, Medical Germany GmbH, Bensheim, Germany).

Data analysis

The results were processed by routine methods of statistical analysis included in STATISTICA v.6.0 (Stat Soft Inc.). Two-tailed Pearson's correlation analysis was performed by Excel 2007 (Microsoft).

RESULTS

Data of spermatozoa' biological parameters are presented in Table 1.

During the 72-hour storage period at 15 °C spermatozoa exhibited a tendency to decrease in motility parameters. The percentage of fast progressive, the percentage of progressive motility and total concentration were the lowest in samples

after storage for 72 hours. However, the differences in sperm concentration, motility and movement of spermatozoa were not significant ($P>0.05$).

During the experiment there were no significant differences between the total sperm motility in ejaculates collected from different animals. The obtained minimum and maximum values were 54.7% and 62.0%, respectively. Nevertheless, our results showed a distinct trend for reduction of the total sperm motility after the 48th hour.

The biochemical patterns of LDH-X and GGT were determined in the proteins of water and Triton X-100 extracts of boar spermatozoa. The data for these parameters are presented in Table 2.

No significant differences were established for the changes in LDH-X activity. There was a wide variation in the individual LDH-X values from 0.74 UI/mg protein to 0.37 UI/mg protein in water extracts, and from 0.21 UI/mg protein to 0.05 UI/mg protein in triton extracts.

Significant differences between GGT activities were found for both extracts between levels at hours 24 and 72, and hours 48 and 72. Differences between hours 24 and 48 were minor in water extract, but significant in triton extract.

Table 1. Sperm concentration and motility in boar ejaculates (n=8) according to the storage time (mean $\pm$ SEM)

Sperm parameters	Storage time (hours)			
	4 h	24 h	48 h	72 h
Sperm concentration ($\times 10^6/\text{mL}$)	288.53 $\pm$ 18.32	285.45 $\pm$ 39.08	269.03 $\pm$ 29.59	243.83 $\pm$ 31.82
Sperm motility				
Fast progressive (%)	57.33 $\pm$ 0.23	56.5 $\pm$ 0.67	56.2 $\pm$ 0.64	54.45 $\pm$ 0.90
Slow progressive (%)	40.65 $\pm$ 0.94	41.73 $\pm$ 0.62	40.85 $\pm$ 1.27	42.03 $\pm$ 1.57
Non-progressive (%)	1.83 $\pm$ 0.76	1.63 $\pm$ 0.81	2.75 $\pm$ 1.29	2.90 $\pm$ 1.78
Progressive motility (%)	59.40 $\pm$ 0.98	57.88 $\pm$ 0.60	58.73 $\pm$ 1.33	56.50 $\pm$ 0.85
Sperm immotility (%)	0.15 $\pm$ 0.10	0.18 $\pm$ 0.12	0.23 $\pm$ 0.11	0.58 $\pm$ 0.44

Table 2. Enzyme activities of boar spermatozoa in water and triton extracts during storage (mean±SEM; n=8)

		LDH-X (UI/mg protein)		GGT (UI/mg protein)	
		Water extract	Triton extract	Water extract	Triton extract
Storage time	24 hours	0.74±0.13	0.05±0.01	0.10 ^a ±0.05	0.06 ^{c,bl} ±0.004
	48 hours	0.55±0.08	0.21±0.05	0.17 ^b ±0.01	0.33 ^{bl,b2} ±0.03
	72 hours	0.37±0.08	0.12±1.00	0.005 ^{a,b} ±0.01	0.001 ^{b2,c} ±0.0007

Values with different superscripts within a column differ significantly (a – P<0.05; b1, b2 – P<0.01; c – P<0.001).

Coefficients of correlation between motility and enzyme activities in boar semen after storage for 72 hours at 15 °C indicated that LDH-X activities seemed to be positively associated with motility in water ($r=0.57$, $P=0.005$) and triton extracts ($r=0.74$, $P=0.0001$). The activities of GGT in water extract ($r=0.84$, $P=0.0001$) was positively associated with motility. However, there was a trend for a weak link between this enzyme and motility in triton extract ($r=0.16$, $P=0.001$). The result showed that with decrease of motility, LDH-X and GGT activities were also decreased.

DISCUSSION

In the present study biological parameters of boar sperm and enzymes level of the lactate dehydrogenase isoenzyme X (LDH-X) and gamma-glutamyl transferase (GGT) were investigated over their storage from 24 to 72 hours at 15 °C.

Sperm motility is the most frequently used parameter to measure boar sperm viability in the ejaculate during and after storage. Indirectly, analyses of sperm motility are expected to provide clues on the potential fertility of the spermatozoa. Unfortunately, the morphological parameters cannot be used alone for predicting sperm quality. Semen characteristics and sperm

morphology are not indicative for boar fertility (Foxcroft *et al.*, 2008; Novak *et al.*, 2010). By using biochemical analyses, we evaluated the biological ability of boar spermatozoa after storage at 15 °C.

The results showed that the 72-hour storage was not associated with appropriate conditions of the gametes, resulting in decreased motility. The high motility of the spermatozoa on the day of semen collection compared with stored semen was logical. It is generally recognised that the fertility potential of stored boar semen is reduced, compared to fresh semen (Rodríguez *et al.*, 2013). In our experiments the total motility of sperms was >60% after 48–72 hours of storage. According to Johnson *et al.* (2000) this level of motility can ensure the optimal fertilising ability of semen.

Although the differences were small, the sperm storage for 24 hours resulted in a higher sperm concentration and higher percent of fast progressive sperm in comparison with semen stored for 72 hours.

Biological semen parameters assessment by SCA is a powerful tool for evaluating the fertilising capacity of boar ejaculates. The detailed motility measurement using SCA in this study provided results, directly applicable in commercial AI centres (Vyt *et al.*, 2004).

The activities of LDH-X and GGT in this study were determined in the proteins of water and Triton X-100 extracts, representing the soluble, sedimentary and membrane forms of the enzymes respectively. The activity of LDH-X (water extract) declined gradually over the period of sperm storage from hour 24 to hour 72. It resulted from the accelerated anaerobic metabolism of the spermatozoa. LDH-X is involved in lactate metabolism and glycolysis of developing and mature spermatozoa. Boar sperm has a sperm-specific isoform of lactate dehydrogenase (LDH-X), mainly located in the principal piece of the tail (Jones & Chanttril, 1989).

Gamma-glutamyl transferase (GGT), believed to be present in the midpiece and acrosomal regions of spermatozoa of certain mammalian species (e.g., the boar) may further affect the GSH content of the oocyte at the time of sperm penetration. Thus, in view of the great number of mitochondria in spermatozoa, these antioxidant mechanisms are important in the maintenance of sperm motility, the rate of hyperactivation, and the ability of sperm to undergo the period storage (Lenzi *et al.*, 1994; Irvine, 1996). Owing to this, the analysis of biochemical parameters could provide further information for the reproductive health and fertility on boars.

When activities of enzymes were plotted against the semen quality parameter motility, interesting associations were observed. Even though significant differences in absolute values for motility at the different period of storage were observed, its association with LDH-X and GGT at each time point (till the 72nd h) underlines the importance of this parameter in predicting fertility. The percentage of motile spermatozoa was positively correlated with LDH-X. Investigations on boar spermatozoa indicated the participation of

LDH-X in a shuttle system utilising the redox couple lactate/pyruvate to transfer H from cytosol to mitochondria, a process known to occur in rat and rabbit spermatozoa (Gallina *et al.*, 1994).

As a result of the storage, the negative correlation between GGT and motility during the period 24–48 h may be a sign for reduced function of enzymatic cell protection against free radicals (Sikka, 1996; Pesch *et al.*, 2006). This is in agreement with the results reported by Rodriguez *et al.* (2013).

The relationships is complicated by the biochemically diverse compartments of the spermatozoa (acrosome, nucleus, mitochondrial-flagellar network), all of which may respond quite differently to storage at 15 °C. The respiratory metabolism of sperm cells has been found to be altered after storage at 15 °C (Ackerman, 1968). The sperm is also exposed to reactive oxygen species (ROS) during their storage through temperature changes (Chatterjee & Gagnon, 2001).

CONCLUSION

The present study on sperm motility and enzyme activities provided valuable information about the status and function of spermatozoa during the 72-hour storage at 15 °C, and allowed predicting the fertilising potential of the semen. The obtained results showed that during the time of storage, the decrease in the spermatozoa with progressive motility was in correspondence with reduced LDH-X and GGT enzyme activity. A stronger correlation was established between LDH-X activity and motility of spermatozoa. We therefore conclude that motility, LDH-X and GGT could be used as biological marker of semen quality after storage for 72 h at 15 °C.

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