Short Paper

Protective role of glutathione in buck semen cryopreservation

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Summary

The aim of this study was to determine the effect of low levels of glutathione on post-thawed buck sperm quality. In this experiment, different concentrations of glutathione [0 (LG-0), 0.5 (LG-0.5), 1 (LG-1), 1.5 (LG-1.5), and 2 (LG-2) mM] were added in a soybean lecithin-based extender. A total of 16 ejaculates from four bucks were collected and pooled. Each pooled sample was divided into five equal parts and each part was diluted by one of the above mentioned groups. After freeze-thawing process, motility and velocity, plasma membrane integrity and functionality, and apoptosis features of spermatozoa were evaluated. The results of this experiment showed that total motility (50.75 ± 2.33), plasma membrane integrity (55.75 ± 3.01) and functionality (46.75 ± 2.79) were higher in LG-1 extender compared to other extenders ($P<0.05$). The percentage of live spermatozoa (53.23 ± 3.26) was higher in LG-1 extender compared to other extenders, with the exception of LG-1.5 extender ($P<0.05$). Also, the percentage of late apoptotic spermatozoa (21.33 ± 1.63) was lower in LG-1 extender compared to other extenders ($P<0.05$). In conclusion, our results showed that GL-1 extender resulted in higher post-thawed buck sperm quality compared to other extenders.

Key words: Buck, Freeze, Glutathione, Lecithin, Semen

Introduction

During freeze-thawing process, spermatozoa are subjected to a series of changes in their environment (Woelders, 1997) that lead to loss of their viability and fertilizing ability (Oehninger *et al*., 2000). It has been well defined that high levels of ROS impair motility and fertilization capacity (Baumber *et al*., 2000; Taylor, 2001). Antioxidants are used as a strategy for decreasing detrimental effect of high levels of ROS in semen freezing extenders (Zanganeh *et al*., 2013; Ghadimi *et al*., 2014; Sharafi *et al*., 2014). In most studies, antioxidants are used in extenders containing animal originated components like milk and egg yolk. There are not enough studies for investigation of antioxidant effects in plant originated component based semen extenders on post-thawed sperm quality.

Glutathione exists in a number of cells and plays an essential role in the defense against oxidative stress. In this way it can react with many ROS and play a cofactor role for glutathione peroxidase. Glutathione peroxidase uses glutathione to reduce hydrogen peroxide to H_2O and lipoperoxides to alkyl alcohols. Also, it has been reported that levels of glutathione were significantly affected by freeze-thawing process (Bilodeau *et al*., 2000) and this process is associated with a significant reduction in glutathione content in sperm (Bilodeau *et al*., 2000).

Therefore, the aim of the present study was to assess the effects of various low concentrations of glutathione supplemented lecithin based extender on post-thawed buck sperm quality.

Materials and Methods

Animals and semen collection

Semen samples (16 ejaculates, four ejaculates in four replicates) were collected from four mature Mahabadi bucks (3–4-year-old) twice a week during the non-breeding season.

Semen extending, freezing and thawing

A soybean lecithin-based extender was used in this study (Salmani *et al*., 2013). Each pooled ejaculate was divided into five equal parts and each part was diluted with one of the following extenders at final concentration of 240×10^6 spermatozoa/ml. In this study, extender was supplemented by different concentrations [0 (LG-0), 0.5 (LG-0.5), 1 (LG-1), 1.5 (LG-1.5), and 2 (LG-2) mM] of glutathione. Diluted semen samples were aspirated into 0.25 ml French straws (IMV, L'Aigle, France) and sealed with polyvinyl alcohol powder. Afterward, straws were frozen in liquid nitrogen (LN) vapor, 4 cm above the LN for 12 min, and then plunged into the liquid

nitrogen for storage.

Sperm motility and velocity parameters

Sperm motility and velocity parameters were assessed by a computer-assisted sperm motility analysis (CASA, Version 12 IVOS, Hamilton-Thorne Bio-sciences, Beverly, MA, USA).

Sperm plasma membrane integrity and functionality

Plasma membrane integrity of spermatozoa was assessed by means of the eosin-nigrosin staining method (Salmani *et al*., 2013). Plasma membrane functionality was assessed by means of hypo-osmotic swelling (HOS) teat based on curled and swollen tails (Salmani *et al*., 2013).

Phosphatidylserine translocation

The percentage of live, early apoptotic, late apoptotic and Necrotic spermatozoa were evaluated using phospatidylserin translocation detection commercial kit according to the manufacturer's instructions (IQP, Groningen, the Netherlands). Each sample was analyzed by flowcytometry (Becton Dickinson, San Khosoz, CA, USA). The sperm samples were classified to four groups (Emamverdi *et al*., 2013):

1) Viable non-apoptotic spermatozoa (A−/PI−)
2) Early-apoptotic spermatozoa (A+/PI−)
3) Late-apoptotic spermatozoa (A−/PI+)
4) Necrotic spermatozoa (A+/PI+)

Statistical analysis

Data were analyzed by GLM procedure using SAS (2002). This study was replicated six times and results were expressed as least squares mean (LSM) ± standard error of mean (SEM).

Results

Sperm motility and velocity parameters

As shown in Table 1, LG-1 extender led to significantly (P<0.05) higher percentage of total motility (50.75 ± 2.33) of spermatozoa compared to other extenders.

Plasma membrane integrity and functionality

The LG-1 extender led to higher (P<0.05) percentages of plasma membrane integrity (55.75 ± 3.01) and functionality (46.75 ± 2.79) compared to the other extenders (Table 2).

Phosphatidylserine translocation

The percentage of live spermatozoa was significantly higher in LG-1 extenders compared to LG-0, LG-0.5 and LG-2 extenders. Also, the percentage of late apoptotic spermatozoa in LG-1 extender was significantly lower compared to other extenders (Table 3).

Discussion

In this present study, total motility, plasma membrane

Table 1: The effect of different extenders on post-thawed buck sperm motility and velocity parameters (LSM ± SEM)

Parameters	Extender					SEM
	LG-0	LG-0.5	LG-1	LG-1.5	LG-2	
TM (%)	32.75^{b}	34.75^{b}	50.75^{a}	43.25^{b}	36.75^{b}	2.33
PM (%)	20.5	22.25	28.25	25.75	20.75	1.77
VAP (μm/s)	83.78	95.15	95.5	94.28	93.28	5.55
VSL (μm/s)	65.83	70.55	70.48	73.85	66.45	4.83
VCL (μm/s)	152.85	166.98	166.33	170.6	165.1	10.62
ALH (μm)	7.3	8.28	8.48	8.15	8.4	0.42
BCF (Hz)	27.2	29.63	28.15	30.13	28.3	1.42
STR (%)	77.5^{a}	71.5^{ab}	72.25^{ab}	75.25^{ab}	68.75^{b}	1.82
LIN (%)	42.75	41.25	42	41.75	39.5	1.35

$^{a, b}$ Different superscripts within rows are significantly different (P<0.05)

Table 2: The effect of different extenders on plasma membrane integrity and functionality of post-thawed buck semen (LSM ± SEM)

Parameters	Extender					SEM
	LG-0	LG-0.5	LG-1	LG-1.5	LG-2	
Plasma membrane integrity (%)	37.75^{b}	35.25^{b}	55.75^{a}	45.25^{b}	39^{b}	3.01
Plasma membrane functionality (%)	29.25^{b}	32^{b}	46.75^{a}	39.25^{b}	35^{b}	2.79

$^{a, b}$ Different superscripts within rows are significantly different (P<0.05)

Table 3: The effect of different extenders on subpopulations of post-thawed buck spermatozoa detected with the Annexin-V (A)/propidium iodide (PI) (LSM ± SEM)

Parameters	Extender					SEM
	LG-0	LG-0.5	LG-1	LG-1.5	LG-2	
Live sperm (%)	35.83^{b}	36.58^{b}	53.23^{a}	50.98^{a}	36.35^{b}	3.26
Early apoptotic sperm (%)	20.67	16.98	17.53	12.48	15.26	1.85
Late apoptotic sperm (%)	36.02^{ab}	38.45^{a}	21.33^{c}	29.05^{b}	40.46^{a}	1.63
Necrotic sperm (%)	7.46	7.97	7.90	7.47	7.91	0.58

$^{a, b, c}$ Different superscripts within rows are significantly different (P<0.05)

integrity and functionality, and viability were higher in LG-1 extender compared to other extenders. Also, LG-1 extender resulted in lower percentage of late-apoptotic spermatozoa compared to other extenders. Like our current result about total motility in LG-1 extender, some researchers (Bucak and Tekin, 2007; Munsi *et al*., 2007) have reported the beneficial effect of glutathione on sperm motility in comparison with control. According to our results, total motility, plasma membrane integrity and functionality, and viability have the same pattern in LG-1 extender. Therefore, part of the beneficial effects of total motility in LG-1 extender may be related to improved plasma membrane integrity and functionality, and viability parameters. On the other hand, our results are not in agreement with previous funding on goat (Salmani *et al*., 2013), buffalo (Ansari *et al*., 2012), and horse (Zhandi and Ghadimi, 2014) semen. They have reported that glutathione has detrimental effects on sperm quality during freezing and cooling condition. This discrepancy might be due to higher concentration of added glutathione in later studies.

In this study, GL-1 extender significantly increased the percentage of sperm plasma membrane integrity and functionality compared to other extenders. It has been demonstrated that glutathione may stabilize the plasma lemma of spermatozoa (Munsi *et al*., 2007). Our results are in agreement with the findings of Bucak and Tekin (2007) who reported that glutathione can improve ram sperm plasma membrane integrity and functionality during the 6 h of liquid storage at 5°C. On the other hand, results are not in agreement with findings of Sharafi *et al*. (2014) who reported that glutathione cannot improve the sperm plasma membrane integrity and functionality. This discrepancy may be due to different concentration of added glutathione.

Finally, the result of Phosphatidylserine translocation assay shows that the proportion of viable spermatozoa is higher in GL-1 extender compared to GL-01, GL-0.5 and GL-2 extenders. Also, the proportion of early apoptotic spermatozoa is lower in GL-1 extender compared to other extenders. These results indicate that glutathione at optimum concentration can help spermatozoa to be rescued from freezing induced apoptosis. Also, the results of this study show similar pattern in the proportions of plasma membrane integrity and functionality, and viability. However, it seems that GL-1 extender can efficiently preserve spermatozoa against apoptosis.

In conclusion, results of the present experiment show that GL-1 extender can efficiently improved some *in vitro* post-thawed ram sperm quality parameters including total motility, plasma membrane integrity and functionality, and viability. However, *in vivo* fertility test to find the best extender is recommended.

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