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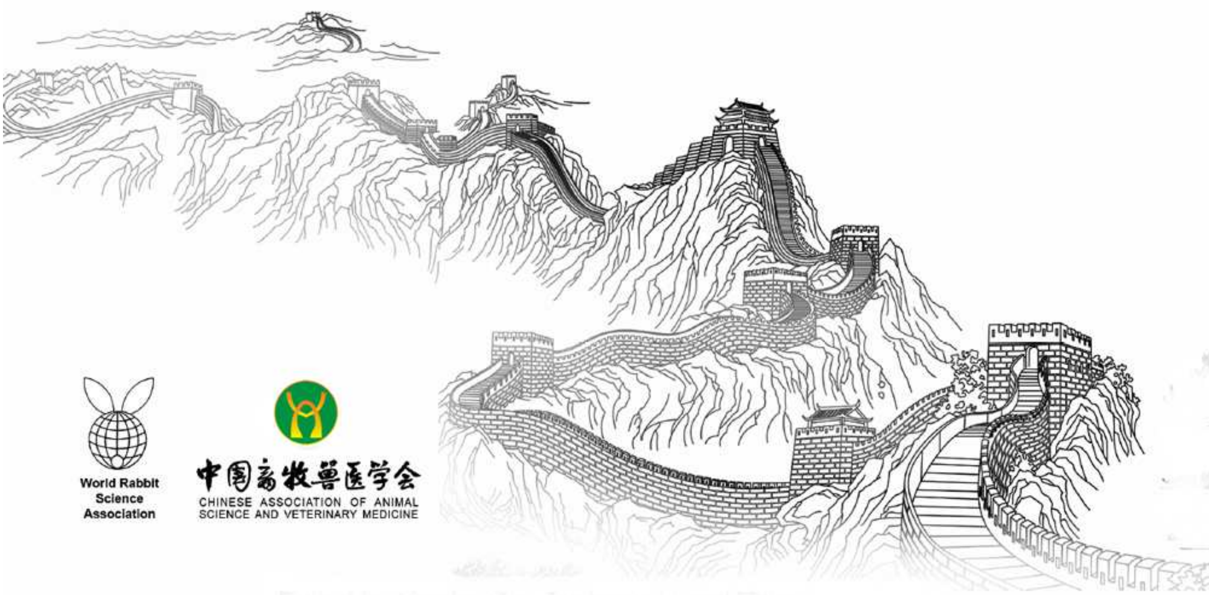

SUPPLEMENTATION OF EXTENDER WITH REDUCED GLUTATHIONE (GSH) PRESERVES RABBIT SPERM QUALITY AFTER CRYOPRESERVATION

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ABSTRACT

The present study aimed to determine the effect of reduced glutathione (GSH) on rabbit sperm quality after freezing and thawing. The pooled ejaculates were used and diluted with freezing extender (TCG with 3.5M and 0.1M sucrose) and supplemented either with 4mM GSH or remained untreated. The samples were cooled, frozen and stored in liquid nitrogen. The post-thaw motility, viability and acrosome were analysed. The results showed that the addition of 4 mM of GSH to the freezing media significantly improved ($P < 0.05$) the progressive motility, total motility and sperm motion characteristics (VSL, VAP, LIN and STR) of rabbit sperm compared to the control. Similarly, the proportions of viable and reacted sperm were different ($P < 0.05$) between 4mM GSH and control. Based upon the results it has been concluded that 4mM glutathione supplementation maintains the sperm motility, viability, and acrosome integrity following the cryopreservation.

Keywords: Glutathione, rabbit sperm, cryopreservation

INTRODUCTION

The processes of cooling, freezing, and thawing produce physical and chemical stress on the sperm membrane that reduce sperm viability and fertilizing ability. The rabbit sperm is peculiar owing to the presence of higher cholesterol content in plasma membrane and hence exhibits greater cold shock resistance during cooling step compared to other domestic species (Darin-Bennett and White, 1977). The higher cholesterol contents guarantee the membrane fluidity (phase transition) which in turn perpetuates the original lipid and protein organization even at lower temperature. In spite of higher resistance to cold shock, the low sperm survival rate after freezing and thawing is still a major constrain in the success of artificial insemination (AI) in rabbit. Therefore, new insights have been made to design different protocols for liquid and frozen storage of rabbit sperm (Theau-Clément *et al.,* 2015). The previous studies demonstrated that the lower molecules cryoprotectants like DMSO and amides provide better protection compared to glycerol and/or ethylene glycol in rabbit (Rosato and Iaffaldano, 2013). Moreover, it has been indicated that the interaction of DMSO and sucrose with membrane phospholipids modifies membrane structure and increase the membrane fluidity and permeability of rabbit sperm during cryopreservation (Moce and Vicente, 2009; Iaffaldano *et al.,* 2012).
All of the above, oxidative stress is the main factor that hinder the sperm quality by provoking excessive ROS production during freezing and thawing. The superfluous of ROS and imbalance between free radicals and antioxidant system of extended semen are related with cold shock and atmospheric oxygen during semen storage and cryopreservation (Cocchia *et al.,* 2011). Albeit, the semen already contains the antioxidants that provides intracellular defence to sperm against the oxidative stress, however, the leakage of sperm cytoplasmic components (antioxidants) to the extracellular environment during semen cryopreservation decreases enzymatic sperm defensive system (Bucak *et al.,* 2008; Gadea *et al.,* 2004). In this context, GSH addition to cryopreservation media has tested in human (Donnelly *et al.,* 2000), bull (Ansari *et al.,* 2014), boar (Giaretta *et al.,* 2015), dog (Ogata *et al.,* 2015), equine (Oliveira *et al*., 2013), ram (Câmara *et al.,* 2011), goat (Gadea *et al.,* 2013), rabbit (Marco-Jimenez *et al.,* 2006) and red deer (Anel-Lopez *et al.,* 2012). The GSH is

one of the major antioxidants present in semen; nevertheless, its level in rabbit sperm is very low compared to the other species (Luberda, 2005). In this background, it was hypothesized that GSH inclusion might preserve the rabbit sperm quality following cryopreservation. Therefore, the present experiment was designed to evaluate the effect of GSH supplementation to the freezing media on rabbit sperm functions after cryopreservation.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study were procured from Sigma Chemical Company (St. Louis, MO, USA)

Animals

The Local Ethical Committee endorsed for the use of experimental animals for the present study. A total of ten adult New Zealand white rabbit bucks (2.5 to 3kg BW, 12 to 18 months) were used in the experiment. Each male was housed in individual cage with ad libitum feed and water supply. Animals were provided the normal daylight throughout the study.

Semen collection and evaluation

The semen samples (100 ejaculates) were collected from 10 rabbits using artificial vagina and gel was removed immediately if present. Semen was collected twice a week from each individual rabbit for successive five weeks. Only the ejaculates milky in colour, having motility more than 70% and concentration ~ 400×10^6/mL, were pooled. The semen samples met the minimal criteria were further used for cryopreservation.

Freezing Protocol

The ejaculates were pooled and after initial observation, the semen was diluted (1:1 v/v) with freezing media (Tris 0.25 M, citric acid anhydrous 88 mM, and glucose 47 mM with 3.5 M DMSO and 0.1 M sucrose) at room temperature and divided into two aliquots. After dilution, one part was supplemented with 4mM GSH whereas, the second part remained untreated. Afterwards, the samples were packaged into French straws and cooled down 5°C with 1hr and equilibrated for further 30 min. Later, the straws were held horizontally above in liquid nitrogen for 10 minutes and then immersed into the liquid nitrogen. The stored straws were thawed and analysed for motility, viability and acrosome reaction.

Post-thaw sperm evaluation

Motility characteristics were determined using a computer-assisted sperm analysis system (CASA: Sperm Class Analyzer® Microptic, Barcelona, Spain). The progressive forward motility (PM, %), total motility (TM, %), curvilinear velocity (VCL, μm/s), linear velocity (VSL, μm/s), average velocity (VAP, μm/s), straightness index (STR, %), linearity index (LIN, %), and oscillation index of the sperm (WOB, %), together with the amplitude of lateral movement of the sperm heads (ALH, μm) and beat cross frequency (BCF, Hz), were recorded.

Acrosome reaction was determined using fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA; 200µg/mL) under an epifluorescence microscope (Olympus-BX53, Hamburg, Germany). The acrosome-reacted sperm emitted the strong green fluorescence whereas unreacted did not show any fluorescence. A total of 200 sperm were counted in each sample.
In order to assess sperm viability, sperm were stained with propidium iodide (PI; 200µg/mL). The sperm heads showed partial or complete pinkish fluorescence were considered nonviable. The sperm without pinkish fluorescence were considered viable. A minimum of 200 sperm per sample were counted for sperm viability.

Statistical analysis

Statistical analysis was performed using the SPSS 17.0 (SPSS Corp., Chicago, IL, USA). Data are presented as percentages and mean ± standard error of the mean (SEM). The independent student *t*-test was applied to compare GSH and control groups.

RESULTS AND DISCUSSION

The results showed that the addition of 4 mM of GSH to the freezing media significantly improved ($P < 0.05$; Table 1) the total motility and progressive motility of rabbit sperm compared to the control. The post-thaw sperm motion characteristics (VSL, VAP, LIN and STR) were significantly higher in the group supplemented with 4 mM of GSH than the control (Table 1). Similarly, the proportions of viable and reacted sperm were different ($P < 0.05$) between the 4mM GSH supplemented and control group following cryopreservation.

Table 1: Post-thaw rabbit sperm quality parameters following freezing in an extender containing 0 or 4mM GSH. (Data is presented as mean ± standard error)

Variables	Control	GSH-4mM	*P*-value
PM (%)	4.6±0.7	7.5±0.9	0.018
TM (%)	28.3±1.6	34.3±1.8	0.02
Viability (%)	11.0±1.3	30.3±2.6	0. 000
Acrosome reaction (%)	82.8±2.6	67.6±2.9	0.001
VCL (µm/Sec)	27.5±1.4	31.8±1.8	0.073
VSL (µm/Sec)	5.6±0.5	7.6±0.6	0.019
VAP (µm/Sec)	12.2±0.9	14.8±1.0	0.07
LIN (%)	20.1±1.5	23.9±0.9	0.043
STR (%)	45.5±1.8	51.1±1.5	0.025
WOB (%)	43.9±2.1	46.6±1.1	0.258
ALH (µm)	1.4±0.2	1.7±0.1	0.217
BCF (Hz)	3.7±0.5	4.9±0.7	0.173

Freezing process impairs the sperm function either by increased production of ROS or by lowering the antioxidant defence system which reduce the in vivo fertilizing capacity (Gadea *et al,* 2004). The increased ROS production during freezing procedure decreases the SOD activity in response to an increment in the superoxide anion production. Similarly, a decrease in GSH content occurs due to decreased in glutathione reductase (GRD) activity and an increase in GSH oxidation by hydrogen peroxide (Gadea *et al.*, 2004). A decrease in intracellular GSH or SOD contents proposed that supplementing the antioxidants to the cryodiluents is a way to improve the post-thaw sperm quality (Gadea *et al.*, 2005). It has been stated that its positive effects are linked to sperm protection against ROS production by maintaining equilibrium between oxidation and reduction of lipids membrane contents (Bansal and Bilaspuri, 2011). However, negative or disadvantageous effects of GSH depend upon the total decrease in enzymatic antioxidant defence during cooling and freezing (Gadea *et al.*, 2011), inappropriate pH or osmolality of freezing extender (Oliveira *et al.*, 2014), concentration of used antioxidants and contact time between sperm and antioxidant during processing (Foote *et al.*, 2002).

Overall the sperm quality in term of motility, viability and acrosome integrity was more in frozen-thawed rabbit sperm in GSH treated group which illustrates the protective effect of GSH on sperm during freezing. The useful results of GSH treatment on post-thaw sperm quality are concomitant with earlier reports in other species (Foote *et al.*, 2002; Gadea *et al.*, 2005; Oliveira *et al.*, 2013; Câmara *et al.*, 2011; Gadea *et al.*, 2013; Anel-Lopez *et al.*, 2012). Whereas, the present findings did not match the previous report of Marco-Jimenez *et al.* (2006) where rabbit sperm were cryopreserved by supplementing the low dose of GSH (0.5mM). The earlier studies showed that the GSH content of bull (Stradaioli *et al.*, 2007), dog (Ogata *et al.*, 2015), human (Gadea *et al.*, 2011) and boar (Gadea *et al.*, 2004) semen is decreased after cryopreservation. However, the rabbit semen already devoid of GSH contents so in turn chances of ROS production become greater compared to other species. A plausible explanation for improved rabbit sperm quality in the present study could be that the GSH supplementation reduces the ROS production either directly using its own SH group or indirectly by acting as a cofactor of the enzymatic antioxidants systems during cryopreservation. Nevertheless, additional studies are needed to prove the beneficial effect of GSH in the improvement of fertility rates *in vitro* and *in vivo* rabbit inseminated with frozen semen.

CONCLUSION

Based upon the current findings it can be concluded that the GSH (4 mM) supplementation in the freezing extender enhances the post-thaw sperm characteristics in rabbit. However, further studies are required to elucidate the fertility rate following insemination with GSH supplemented frozen-thawed semen in rabbit. .

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