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GLUTATHIONE SUPPLEMENTATION IN SEMEN EXTENDER IMPROVES RABBIT SPERM ATTRIBUTES DURING REFRIGERATION

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Abstract: In the present study, we evaluated the sustaining effect of various glutathione (GSH) concentrations in extender on rabbit sperm attributes during storage at 5°C for 24 h. Semen was collected from regular donor rabbit bucks using an artificial vagina and initially evaluated for sperm quality. The qualifying ejaculates were diluted with one of the extenders having 0, 1, 2, 4 or 8 mM GSH, to achieve a final concentration of 1×10^8 sperm/mL. The extended samples were stored at 5°C for 24 h. Sperm motility, motion kinetics, acrosome integrity and viability were assessed after 3, 6, 12 and 24 h of storage. The results showed that total sperm motility and sperm motion kinetics (oscillation index of the sperm, straightness index and beat cross frequency) were influenced ($P<0.05$) by glutathione dose and refrigeration time. An interaction of ($P<0.05$) GSH concentrations and refrigeration time was observed for sperm viability and acrosome reaction rate. In conclusion, the 4 mM GSH supplemented extender's protective influence was remarkable to maintain rabbit sperm quality for 24 h 5°C.

Key Words: glutathione, refrigeration, sperm quality, rabbit.

INTRODUCTION

Semen storage processes such as refrigeration and cryopreservation induce physical and chemical stress in the sperm membrane, resulting in reduced post-thaw sperm survival. The cryopreservation of rabbit sperm is still challenging in the rabbit production industry; therefore, freshly diluted or cooled semen is preferred for insemination (Nagy *et al.,* 2002). Generally, rabbit sperm can withstand cold shock better compared to the sperm of other domestic species due to a high level of cholesterol content in the plasma membrane (Darin-Bennett and White, 1977). Long-term cold storage of rabbit sperm steadily deteriorates the rabbit sperm quality (Johinke *et al.,* 2014) and fertilising capacity by excessive liberation of reactive oxygen species (ROS), e.g. superoxide anion radical (O^{2-}), hydrogen peroxide (H_2O_2) and lipid hydroperoxides (Bansal and Bilaspuri, 2011). Atmospheric oxygen is an additional factor that exacerbates ROS production during semen refrigeration. The semen dilution and damage to the sperm plasma membrane lead to a reduction in the physiological antioxidant system of semen (Saraswat *et al.,* 2016). To overcome the condition, the supplementation of antioxidants, physiological and non-physiological, has been tested in different species to protect the sperm against the harsh impact of oxidative stress imposed during the cooling, freezing and thawing process (Bansal and Bilaspuri, 2011; Yimer *et al.,* 2016).

Supplementation with glutathione (GSH) protects the sperm against oxidative stress by inactivating the ROS during refrigeration (Ansari *et al.,* 2011; Sarangi *et al.,* 2017; Zhandi and Ghadimi, 2014; Zeitoun and Al-Damegh, 2015;). The ROS detoxification is brought about by reverse reaction of GSH (GPX-GSSG). GSH is amongst the major

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antioxidants present in semen in different species; nevertheless, its level in rabbit sperm is very low comparatively (Luberda, 2005). In this context, the exogenous GSH inclusion in semen extender has been tested to observe its capacity for the long-term maintenance of sperm quality. The present study was designed to determine the effect of different GSH supplementation concentrations on rabbit semen held at 5°C for 24 h.

MATERIALS AND METHODS

Chemicals

All the used reagent and chemicals were purchased from Merck (Darmstadt, Germany) except GSH and glucose (Sigma Chemical Company, St. Louis, MO, USA).

Animal management

The study was conducted from February to April (spring season). Prior to execution of the experiment, approval was granted by the Local Ethical Committee for the use of experimental animals. A total of ten adult ($n=10$) New Zealand white rabbit bucks (2.5 to 3 kg body weight, 12 to 18 mo) were used. Each male was caged individually with *ad libitum* supply of feed and water. Natural daylight was provided in a ventilated room.

Semen collection and initial evaluation

Semen was collected using an artificial vagina and initially evaluated for sperm quality. A total of 33 ejaculates, with normal colour, 0.4 to 0.5 mL volume, >80% motility and ~400×10^6/mL concentration, were collected from 10 male rabbits. The semen ejaculates were pooled to avoid individual effect and six observations were made during the trial. Initially, the pooled semen was diluted with TCG extender (0.25 M Tris, 88 mM citric acid anhydrous and 47 mM glucose) at ratio of 1:2 and observed for motility kinetics, viability and acrosome integrity (Naseer *et al.,* 2020).

Semen refrigeration

The diluted samples were split into five aliquots according to GSH concentration [0 (control), 1, 2, 4, 8 mM]. The final aliquot volume (1 mL) at 1×10^8/mL sperm concentration was achieved by adding TCG extender. The samples were kept in a cold cabinet for a period of 24 h at 5°C and monitored for sperm motility, sperm kinetics, viability and acrosome integrity at 3, 6, 12 and 24 h of storage.

Semen evaluation

Sperm motility characteristics were determined using a computer-assisted sperm analysis system (CASA; Sperm Class Analyzer-SCA®, Microptic S.L. Viladomat, Barcelona Spain). The CASA system is based on the analysis of 25 consecutive digital images, taken from a single field at a magnification ×100 against a dark background in a 1 s time lapse. The sample aliquots (3 µL) were placed on a pre-warmed slide and viewed under phase contrast microscope. Four or five separate fields were chosen for each sample and a minimum of 500 sperm per sample were analysed. The progressive motility (PM, %), total motility (TM, %), curvilinear velocity (VCL, µm/s), straight linear velocity (VSL, µm/s), average pathways velocity (VAP, µm/s), straightness index (STR, %), linearity index (LIN, %) and oscillation index of the sperm/wobble (WOB, %) were recorded, together with the amplitude of lateral movement of the sperm heads (ALH, µm) and beat cross frequency (BCF, Hz).

To assess sperm viability, the sperm were stained with propidium iodide (PI; 200 µg/mL). Sperm viability was estimated using differential staining as revealed by sperm membrane integrity differences. The PI penetrated only sperm with damaged membrane and showed the partial or complete pinkish fluorescence that indicated dead sperm, whereas the live sperm exhibited no fluorescence over the sperm head. A minimum of 200 sperm per sample were counted.

Fluorescein isothiocyanate-conjugated *peanut agglutinin* (FITC-PNA; 200µg/mL) staining procedure was used for acrosomal integrity. A total of 200 sperm were counted per sample, using epifluorescence microscopy (Olympus-BX53, Hamburg, Germany) to differentiate the reacted and non-reacted sperm. The sperm with damaged acrosome emitted

Table 1: Initial seminal characteristics after dilution of semen, collected from ten (n=10) experimental rabbits.

Parameters	Values
Progressive motility (PM; %)	55.6±2.3
Total motility (TM; %)	83.5±1.3
Curvilinear velocity (VCL; μm/s)	83.4±3.0
Straight linear velocity (VSL; μm/s)	27.5±1.1
Average pathways velocity (VAP; μm/s)	45.0±1.7
Wobble (WOB; %)	59.9±1.1
Linearity index (LIN; %)	54.5±0.6
Straightness index (STR; %)	45.9±1.3
Amplitude of lateral movement of sperm head (ALH; μm)	4.2±1.6
Beat cross frequency (BCF; Hz)	12.0±0.4
Sperm viability (%)	74.3±6.6
Acrosome reaction rate (%)	4.6±0.4

the strong apple green fluorescence under a fluorescence microscope, whereas non-reacted sperm did not show any fluorescence.

Statistical analyses

Statistical analyses were performed using the SPSS 17.0 (SPSS Corp., Chicago, IL, USA). Data are presented as least squares mean±standard error of the mean. Data were tested for normality (UNIVARIATE procedure) and microscopic sperm variables at different time periods between control and GSH supplemented groups were analysed using repeated measures ANOVA. The specific sperm treatment (GSH concentrations) and the time points of refrigeration were the main effects and their interaction was also included in the model. Differences were considered statistically significant at $P<0.05$.

RESULTS

The initial sperm variables after dilution were up to desired quality and are presented in Table 1. The effect of different GSH concentrations on sperm motility and sperm kinetics characteristics during 24 h storage period is presented in Table 2. The progressive motility was affected by refrigeration time ($P<0.05$), whereas, the total sperm motility was influenced by both glutathione dose ($P<0.05$) and refrigeration time ($P<0.05$). The kinetics of each sperm motion were different ($P<0.05$) in response to refrigeration time. The glutathione dose affected ($P<0.05$) only WOB, STR and BCF sperm motion kinetics variables during cool storage.

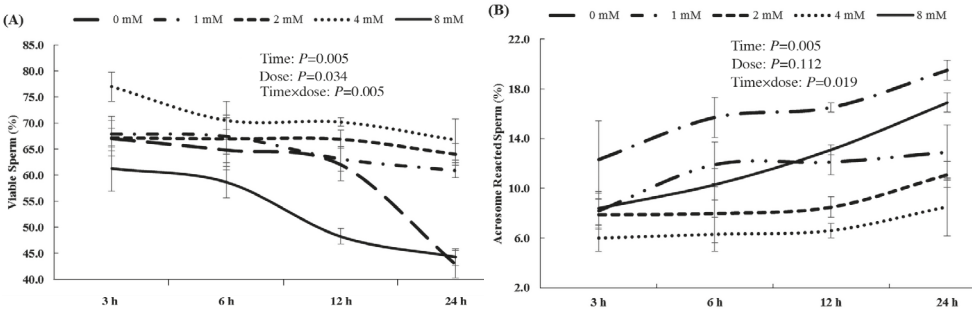

Figure 1: Effect of various doses of glutathione (0, 1, 2, 4 and 8 mM) on rabbit sperm viability (A) and acrosome reaction rate (B) during 24 h storage at 5° C.

Table 2: Effect of glutathione supplemented semen extender on sperm motility and sperm motion kinetics of rabbit sperm stored at 5°C for 24 h.

	Glutathione dose (mM)					Refrigeration time (h)			
Variables	0	1	2	4	8	3	6	12	24
Progressive motility (PM; %)	29.4±3.5	30.4±3.4	37.0±4.3	42.0±4.8	30.9±4.2	49.8±3.8^{b}	38.3±3.3^{ab}	31.0±2.2^{ab}	16.7±2.0^{a}
Total motility (TM; %)	67.2±3.6^{xy}	69.0±3.3^{xy}	72.0±2.8^{xy}	75.9±3.1^{y}	63.4±3.7^{x}	82.3±1.9^{d}	73.5±2.3^{b}	68.5±2.4^{b}	53.9±2.4^{a}
Curvilinear velocity (VCL; μm/sec)	56.8±4.5	58.7±3.5	60.7±4.0	69.7±5.4	59.3±5.8	79.8±4.2^{d}	67.8±3.4^{c}	57.0±2.5^{b}	39.5±1.8^{a}
Straight linear velocity (VSL; μm/sec)	18.3±1.9	17.4±1.4	19.9±1.7	21.8±2.1	16.3±2.1	25.8±1.5^{d}	22.2±1.3^{c}	17.2±1.1^{b}	9.7±0.7^{a}
Average pathways velocity (VAP; μm/sec)	29.5±2.8	29.6±2.1	32.0±2.4	36.2±3.4	29.5±3.3	42.9±2.4^{b}	35.9±1.9^{b}	29.1±1.7^{b}	17.6±1.1^{a}
Wobble (WOB; %)	34.0±2.3^{y}	32.9±2.3^{y}	36.1±2.1^{y}	34.6±2.2^{y}	25.8±1.1^{x}	35.5±1.8^{b}	35.2±1.8^{b}	33.0±1.9^{b}	27.2±2.0^{a}
Linearity index (LIN; %)	53.4±3.0	52.0±2.6	55.6±2.7	53.5±2.9	53.0±1.3	55.5±2.2^{b}	56.2±2.0^{b}	54.0±2.2^{ab}	48.3±2.3^{a}
Straightness index (STR; %)	51.7±1.5^{xy}	52.2±1.5^{xy}	54.0±1.4^{y}	53.3±1.4^{y}	48.2±1.1^{x}	54.5±0.8^{b}	54.4±1.0^{b}	51.8±1.1^{b}	46.9±1.5^{a}
Amplitude of lateral movement of sperm head (ALH; μm)	2.2±0.1	2.3±0.09	2.1±0.08	2.4±0.09	2.2±0.1	2.5±0.06^{c}	2.5±0.08^{c}	2.2±0.06^{b}	1.7±0.06^{a}
Beat cross frequency (BCF; Hz)	10.0±0.8^{y}	9.5±0.8^{y}	11.0±0.8^{y}	11.5±0.6^{y}	6.9±0.9^{x}	11.0±0.6^{b}	11.7±0.7^{b}	10.4±0.6^{b}	6.0±0.6^{a}

Means in a row not sharing supercripts show significant differences (P<0.05) for glutathione does (x,y) or refrigeration time (a,b,c,d).
Data are expressed as means±standard error means.

There was a significant effect of GSH concentrations (P<0.005) and refrigeration time (P=0.034) on sperm viability and their interaction was significant (P=0.005; Figure 1A). The acrosome reaction was only affected (P=0.005) by chilling duration and an interaction of glutathione dose and chilling time was significant (P=0.019; Figure 1B).

DISCUSSION

The refrigeration and cryopreservation process usually hampers sperm quality by inducing excessive ROS production. The sperm membrane is the main organelle that is exposed to the challenging conditions of cooling and freezing; the membrane protection is thus of prime importance during processing, especially against ROS (Bansal and Bilaspuri, 2011). Sperm has a well-defined antioxidant system to combat the oxidative stress; however, the dilution, cooling and freezing process reduces the antioxidant potency against ROS. Therefore, the exogenous supplementation of antioxidants in semen diluents is considered a beneficial practice to improve sperm quality (Petruska *et al.,* 2014). In this sense, the aim of the present study was to identify the optimal GSH concentration to protect rabbit sperm during a storage period at 5°C.

To our knowledge, few studies have been conducted for long-term preservation of rabbit sperm at 5°C by using different extenders in combination with exogenous antioxidants (Rosato and Iaffaldano, 2011; Rosato *et al.,* 2012; Sarıözkan *et al.,* 2013; Sarıözkan *et al.,* 2014; Johinke *et al.,* 2015; Maya-Soriano *et al.,* 2015). In the present study, we observed an improvement in motility, viability and acrosomal integrity of rabbit sperm by supplementing a medium dose of GSH (4 mM) in extender during 24 h of liquid storage. These findings are in agreement with other studies in different species (Munsi *et al.,* 2007; Ansari *et al.,* 2011; Câmara *et al.,* 2011; Zhandi and Ghadimi, 2014; Sarangi *et al.,* 2017). The semen of other species contains variable levels of GSH; therefore, variability in sperm quality following GSH supplementation could be an observable fact. In the present study, improvement in sperm quality could be explained saying that low levels of antioxidants restore the equilibrium by scavenging the amounts of ROS produced, sequentially maintaining the metabolic activity of the sperm during refrigeration (de Lamirande and Gagnon, 1995).

Holding the sperm for 24 h at 5°C in control group resulted in poor quality, which reflects the action of ROS

production and related damage to the sperm. Similarly, the high dose of GSH showed an inadequate response for the sperm quality after refrigeration. These findings are inconsistent with earlier studies (Munsi *et al.,* 2007; Ansari *et al.,* 2011; Câmara *et al.,* 2011; Zhandi and Ghadimi, 2014). The damaging effects of high concentrations of GSH on the sperm quality have also been described: decreased plasma membrane integrity, degrading the sperm DNA (Prete *et al.,* 2018), damaging the acrosome (Ansari *et al.,* 2011), reducing the pH and increasing the medium viscosity (Oliveira *et al.,* 2014). Moreover, Peña *et al.* (2003) stated that the action of any antioxidants is dependent on the type and dose used against ROS production. The current study clearly demonstrates that rabbit sperm contains a weak antioxidant system, and readily undergoes oxidation processes during aerobic conditions. Medium doses (2 to 4 mM) of GSH addition are beneficial to sperm under refrigeration for several hours in an extender, especially with low osmolality.

Cooling and freezing processes impair the sperm function either by increased production of ROS or by lowering the antioxidant defence system, which in turn reduces the *in vivo* fertilising capacity of sperm (Petruska *et al.,* 2014). The increased ROS production during freezing procedure lowers the superoxide dismutase (SOD) activity in response to an increment in the superoxide anion production. Similarly, a reduction in GSH content leads to decreased glutathione reductase activity and increased GSH oxidation by hydrogen peroxide (Prete *et al.,* 2018). The decrease in intracellular GSH or SOD contents suggested that supplementing the antioxidants to the diluents could be a strategy to improve sperm quality during semen preservation procedures (Petruska *et al.,* 2014). The exogenous GSH provision in semen diluters is related to the sperm protection against ROS production by maintaining equilibrium between oxidation and reduction of lipids membrane contents (Bansal and Bilaspuri, 2011). However, the impact of GSH supplementation relies on the total decrease in enzymatic antioxidant defence during cooling and freezing (Prete *et al.,* 2018), the inappropriate pH or osmolality of the used extender (Oliveira *et al.,* 2014), the concentration of antioxidants used or the contact time for sperm and antioxidant during processing (Munsi *et al.,* 2007). In the case of rabbit semen, the type of diluents (Fadl *et al.,* 2019; 2020) used plays an important role, as excessive dilution of seminal plasma deteriorates the sperm quality during cooled storage (Aksoy *et al.,* 2008; Domingo *et al.,* 2018). A plausible explanation for improved rabbit sperm quality in the present study is that the GSH supplementation reduces ROS production directly using its own SH group, or indirectly by acting as a cofactor of the enzymatic antioxidants systems during cooling. Nevertheless, additional studies are needed to prove the beneficial effect of GSH in improving fertility rates in *in vitro* and *in vivo* rabbits inseminated with frozen semen.

Based on the current findings, it is summarised that the GSH supplementation in extenders provides beneficial effects on rabbit sperm quality during the refrigeration and a dose of 4 mM GSH is the most appropriate to protect the sperm during long-term cold storage.

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Notes on contributors: Melih Aksoy designed the experiment, review and editing; Zahid Naseer and Ejaz Ahmad executed the trials, analysed the data and drafted the manuscript.

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