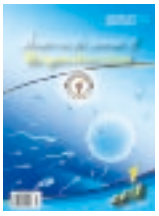

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Effects of reduced glutathione on Boer goat semen freezability

Rawash ZM[1], Ebtihal A Ibrahim[2], El-Raey M[3]✉

[1]*Artificial Insemination and Embryo Transfer Department, Animal Reproduction Research Institute, Al Haram*

[2]*Biology of Reproduction Department, Animal Reproduction Research Institute, Al Haram*

[3]*Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, P.O: 13736, Tokh, Kaliobia, Egypt*

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ABSTRACT

Objective: To evaluate the effects of reduced glutathione on the quality of cryopreserved Boer buck spermatozoa. **Methods:** The current study was carried out on five Boer bucks from which semen samples were collected by artificial vagina. After microscopical evaluation at 37 ℃, semen samples that fulfill the ideal requirements for extension were diluted in a tris–based extender including different concentrations of reduced glutathione (2, 5, 7 and 10 mM) and those without glutathione served as a control. Sperm motility, viability, acrosome integrity, DNA integrity, total antioxidant capacity and lipid peroxidation were assessed post-thawing. **Results:** The current results revealed that post-thawing motility, viability and acrosomal integrity were significantly improved [(66.67±5.50)%, 168.30±18.59 and (12.75±2.45)%, respectively] when 5 mM glutathione was added to semen extender; especially as compared with the control [(40.00 ±2.88)%, 95.00±8.90 and (25.75±3.46)%, respectively]. Similarly, at this concentration (5 mM) sperm DNA damage, tail length and tail moment of cryopreserved semen were significantly (P<0.05) reduced [(2.32±0.27)%, (1.64±0.49) μm and 3.55±0.63, respectively] compared with the control extender [(6.66±0.84)%, (4.09±0.47) μm and 26.47±0.51, respectively]. Moreover, addition of 5 mM glutathione to buck semen extender significantly (P<0.05) increased total antioxidant capacity [0.51±0.07) mμ/mL] and decreased lipid peroxidation of cryopreserved spermatozoa [(8.68±2.72) nmol/mL] compared with the control [(0.18±0.02) mμ/mL and (24.92±5.80) nmol/mL, respectively]. **Conclusions:** The addition of 5 mM glutathione to semen diluent improve freezability of Boer buck spermatozoa through DNA protection from deterioration and oxidative stress reduction. Moreover, 10 mM of glutathione exerts cytotoxic effects on Boer buck semen.

1. Introduction

Goats are considered as one of important backbone blocks in the economy of rural countries like Egypt, as they are chief source for meat, milk and skin. So, all goats, producing countries shall intend to increase this animal population by all possible means. It is clear that artificial insemination and its related assisted reproductive technologies are considered the most important of these applications. Goat's artificial insemination can achieve widespread progression of buck semen, limiting the spread of sexually transmitted diseases and

✉Corresponding author: El-Raey M, Department of Theriogenology, Faculty of Veterinary Medicine, Benha University, P.O: 13736, Tokh, Kaliobia, Egypt.
E-mail: elraay_310@yahoo.com
Tel: +201003828279

First author: Rawash ZM, Artificial Insemination and Embryo Transfer Department, Animal Reproduction Research Institute, Al Haram.

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facilitating genetic improvement programs in goat's industry.

Semen manipulation during cryopreservation is associated with a plethora of changes in spermatozoa such as capacitation like effects[1], reduction in integrity of the plasma and acrosomal membranes[2], diminution in motility and ability to penetrate oocyte *in vitro*[3]. Moreover, cryopreservation leads to a significant reduction in the level of spermatozoa antioxidants[4], predisposing the sperm to the generated reactive oxygen species (ROS) such as superoxide radical, hydroperoxyl radical and hydroxyl radical[5], which cause sperm cell damage[6]. Generally, there is a state of equilibrium between ROS production and their scavenging systems which are delicate. This balance can be distrusted by excessive centrifugation and freezing/thawing processes during cryopreservation resulting in excessive ROS production in sperm-processing media[7]. ROS excess during cryopreservation has been coupled with reduced post-thaw motility[8], mainly due to changes in membrane transportation[9]. Additionally, excessive ROS results in reduction in the sperm viability, membrane integrity and other sperm functions[8]; which in turn affects the sperm fertilizing potentials. Therefore, the amount of ROS should be restricted to the minimum requirements to preserve the cell functions[6]. The sperm cell membrane contains high levels of polyunsaturated fatty acids contents that are easily damaged by ROS through the process of peroxidation[10-12]; the latter is considered as bio-index or a key to evaluate the degree of sperm membranes damage by the excessive ROS[13], which in turn affects the future fertility characteristics of semen.

Sperm motility, viability and fertilizing ability can be improved or preserved by addition of various motility enhancing agents or antioxidants in semen diluent. The antioxidants can neutralize or reduce the risk of damage to spermatozoa during cryopreservation process by combating harmful effects of ROS or enhancing its antioxidant enzymes[14,15]. In between, glutathione which is considered as a thiol tripeptide (γ -glutamyl cysteinyl glycine) has antioxidative potentials and can maintenance the intracellular redox conditions[16,17]. Glutathione is considered as a natural reservoir of redox force depending upon its sulphydryl group in reduced glutathione (GSH) and oxidized glutathione forms, which quickly protects different cell types against oxidative stress[18]. Furthermore, glutathione has roles in protein synthesis regulation, cellular detoxification, and leukotriene synthesis depending on its components from glutamate, cysteine, and glycine amino acids[17]. In semen, glutathione plays the same roles against the excessive generated ROS during centrifugation process that removes its seminal plasma containing antioxidant, also during the process of freezing/thawing[8,16,19]. Furthermore, glutathione plays a cofactor role for glutathione peroxidase which uses glutathione to reduce hydrogen peroxide to H_2O and lipoperoxides to alkyl alcohols[15], even though it has been found that cryopreservation process reduced the content of GSH of spermatozoa and seminal plasma[8,16]. Therefore, depending on the scarcity of information regarding the effects of GSH on Boer buck spermatozoa, the current study is planned to investigate the effects of GSH on Boer buck semen freezability, then to maintain the qual ity of buck semen and enhance its fertilizing potentials.

2. Materials and methods

2.1. Animals and semen collection

Semen samples from 5 mature fertile Boer bucks (2 years old) were collected by artificial vagina twice weekly. Only semen samples of at least 80% initial motility and 3×10^9 sperm/mL were used. The spermatozoa were separated from seminal plasma through twice centrifugation (10 min/2 000 rpm). Pelleted sperms were re-suspended with semen extender and pooled, split into 5 portions and diluted at 30 ℃ with Tris-based extender (6% glycerol and 20% egg yolk), and supplemented with different concentrations of glutathione (2, 5, 7 and 10 mM) and those without glutathione served as a control. The extended semen was cooled to 5 ℃ throughout 60 min in a cold cabinet and equilibrated at 5 ℃ for a period of 2.5 h. After equilibration, the cooled semen was loaded in straws (IMV, L'Aigle, France/0.25 mL), then exposed to liquid nitrogen vapor (6 cm above liquid nitrogen) / 15 min. Finally, the straws were stored in the liquid nitrogen till analysis. Frozen straws were thawed in a water bath at 37 ℃/30 s. Post-thawing viability, acrosomal integrity and motility were assessed according to Salamon and Maxwell[2].

2.2. Biochemical analysis

Aspartate-aminotransferase, alanine-aminotransferase and alkaline phosphatase enzyme concentrations were measured according to Burtis *et al*[20] to estimate membrane stabilizing roles of GSH. Additionally, lipid peroxidation was estimated by malondialdehyde (MDA) generation that was determined by thiobarbituric acid test as described by Cortossa *et al*[21].

2.3. Assessment of sperm DNA integrity

DNA integrity and the incidence of DNA fragmentation were detected using neutral single cell gel electrophoresis assay (comet assay) according to Boe-Hansen *et al*[22]. Frozen-thawed spermatozoa were diluted in phosphate buffer saline, embedded in agarose, and then subjected to lysis, DNA decondensation, electrophoresis, and finally DNA was stained with 50 μL of 20 μg/mL ethidium bromide (Sigma). Stained sperms were visualized by fluorescent microscope. Compact and bright fluorescent appearance of the sperm heads in the comet assay denotes its intact nuclei, while its DNA fragmentation was denoted by a tail behind its head due to DNA migration during the electrophoresis giving comet appearance[23]. To assess the comet assay results, software computer program was used to judge the sperm nuclei.

2.4. Statistical analysis

Costat Cottort software program (1986), version 3.03, was used to analyze the current data. Results were expressed as means ± SEM. Least significant difference at 1% (P<0.01) and 5% probability (P<0.05) was used to compare values.

3. Results

Table 1 illustrated that addition of GSH to Boer buck semen extender improved its freezability compared to control group, in a dose-dependent pattern. Where, 5 mM of glutathione in Boer buck semen extender appeared to be the best concentration that significantly increased (P<0.05) its post-thawing sperm motility, viability index and acrosomal integrity [(66.67±5.50)%, 168.30±18.59 and (12.75±2.45)%, respectively] compared with control semen samples [(40.00 ±2.88)%, 95.00±8.90 and (25.75±3.46)%, respectively]. Moreover, 10 mM of glutathione seemed to be cytotoxic to Boer buck semen; where the viability index was lowered 89.00±15.99 compared with the control 95.00±8.90.

Table 1

Effect of GSH at different concentrations on Boer buck semen freezability (mean ± SEM).

Treatment	Post-thawing motility (%)	Viability index*	Acrosomal integrity (%)
Control	40.00±2.88^{b}	95.00±8.90^{b}	25.75±3.46^{a}
2 mM glutathione	52.75± 4.75^{ab}	135.13±18.95^{ab}	18.00±4.34^{ab}
5 mM glutathione	66.67±5.50^{a}	168.30±18.59^{a}	12.75±2.45^{b}
7 mM glutathione	58.25±5.15^{ab}	133.88±16.10^{b}	16.00±3.16^{ab}
10 mM glutathione	45.50±11.50^{b}	89.00±15.99^{b}	26.50±4.72^{a}

Three replications at least of the experiment were conducted.

^{abc}superscripted values were significantly different at (P<0.05). *Viability index = (post-thawing M÷2 +M/1 h + M/2 h + M/3 h) where, M/ refer to motility after certain time.

Table 2 clarified that 5 mM glutathione addition in Boer buck semen extender resulted in significant reduction of aspartate-aminotransferase, alanine-aminotransferase and alkaline phosphatase enzyme concentrations [(55.50±1.72) U/L, (19.75±2.46) U/L, (20.66±3.40) U/L, respectively] compared with the control extender [(100.50±7.75) U/L, (26.75±2.10) U/L and (26.45±2.88) U/L, respectively]. Moreover, 5 mM of GSH in Boer buck semen extender significantly (P<0.05) increased the total antioxidant capacity and reduced lipid peroxidation of frozen-thawed buck semen [(0.51±0.07) mμ/mL and (8.68±2.72) nmol/mL, respectively] compared with the control semen [(0.18±0.02) mμ/mL, and (24.92±5.80) nmol/mL, respectively].

Table 3 and Figure 1 show the influence of glutathione addition to the semen extender on the DNA integrity of the frozen-thawed Boer buck spermatozoa. The present data pointed that 5 mM glutathione in Boer buck semen extender significantly (P<0.05) decreased its DNA fragmentation percent, tail length and tail moment after freeze-thawing processes [(2.32±0.27)%, (1.64±0.49) μm and 3.55±0.36, respectively] compared with GSH free extender [(6.66±0.84)%, (4.09±0.47) μm and 26.47±0.51, respectively].

Table 3

Effect of glutathione at different concentrations on DNA integrity of Boer buck spermatozoa (mean ± SEM).

Treatment	DNA integrity (%)	Tail length(μm)	Tail moment*
Control	6.66±0.84^{a}	4.09±0.47^{a}	26.47±0.51^{a}
2 mM glutathione	3.36±0.50^{bc}	4.11±0.46^{a}	10.80±0.80^{b}
5 mM glutathione	2.32±0.27^{c}	1.64±0.49^{b}	3.55±0.36^{c}
7 mM glutathione	3.53±0.98^{bc}	2.30±0.47^{b}	7.48±1.72^{bc}
10 mM glutathione	5.23±0.23^{ab}	4.37±0.64^{a}	22.76±3.21^{a}

Three replications at least of the experiment were conducted.

^{abc} Superscripted values were significantly different at P<0.05.

*Tail moment could be calculated by multiplying DNA fragmentation % by tail length.

Table 2

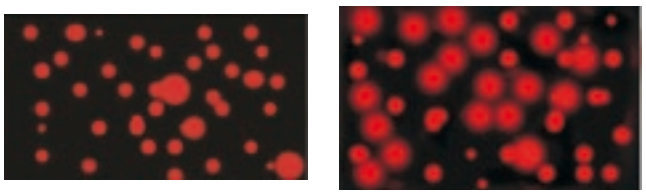
Effect of GSH at different concentrations on Boer buck semen biochemical activity.

Treatment	AST (U/L)	ALT (U/L)	ALP (U/L)	TAC (mμ/mL)	MAD (nmol/mL)
Control	100.50±7.75^{a}	26.75±2.10^{a}	26.45±2.88^{a}	0.18±0.02^{b}	24.92±5.80^{a}
2 mM glutathione	95.65±4.25^{a}	20.75±2.34^{ab}	19.38±3.74^{b}	0.38±0.04^{a}	18.25±1.94^{ab}
5 mM glutathione	55.50±1.72^{c}	19.75±2.46^{b}	20.66±3.40^{ab}	0.51±0.07^{a}	8.68±2.72^{b}
7 mM glutathione	65.00±8.92^{bc}	17.25±1.75^{b}	17.13±1.65^{b}	0.35±0.06^{a}	15.67±4.65^{ab}
10 mM glutathione	98.50±7.18^{a}	24.00±2.75^{ab}	21.88±4.17^{ab}	0.19±0.04^{b}	20.65±2.91^{a}

Three replications at least of the experiment were conducted.

^{abc} superscripted values were significantly different at (P<0.05).

AST: Aspartate-aminotransferase; ALT: Alanine-aminotransferase; ALP: Alkaline phosphatase; TAC: Total antioxidant capacity.



A B

Figure 1. Single cell gel electrophoresis.

A: low DNA fragmentation (%) that was illustrated by limited amount of DNA in the comet tail of Boer buck spermatozoa cryopreserved in tris extender suplemented with 5 mM glutathione.

B: high DNA fragmentation (%) that was illustrated by huge amount of DNA in the comet tail of Boer buck spermatozoa cryopreserved in tris extender without glutathione.

4. Discussion

Assisted reproductive techniques such as artificial insemination are used in goat's industry with the objectives of increasing its genetic gain and producing livestock with improved reproductive efficiency. Despite these advantages, artificial insemination in goats is poorly applied due to mediocre outcomes when frozen/thawed spermatozoa are used. Moreover, few artificial insemination records are conducted worldwide using frozen–thawed Boer buck semen. Therefore, there is an interest in the use of this technology to enhance Boer buck semen industry.

When spermatozoa were frozen / thawed, they were subjected to various stressors (physical, physiological, osmotic and chemical) that resulted in disruption of the transbilayer phospholipids asymmetry of mammalian sperm, thus, damaging the plasma membrane, increasing its susceptibility to lipid peroxidation[7,24–27], predisposing the mammalian sperm to gross morphologic damage and decreasing motility and fertilizing capability. The current results explain that GSH supplementation to Boer buck semen extender improved its freezability. These results are in harmony with those of Sinha *et al*[14], Foote *et al*[28], Badr *et al*[29] and Badr *et al*[30], who confirmed obvious increase in post-thaw motility and acrosomal integrity of buck and bovine spermatozoa when GSH was added to the semen extender. GSH protective effects on sperm fertility characteristics were dose-dependent. The current study declared that addition of 5 mM glutathione into the semen diluent was satisfactorily effective to improve the freezability of Boer buck spermatozoa and these results comes in harmony with the study of Sinha *et al*[14]. On the other hand, the current results run in disagreement with Noei Razliqi *et al*[15] and Sarangi *et al*[31] who reported 1mM; while Badr *et al*[30] reported 2 mM and Foote *et al*[28] reported 0.05-1.00 mM of glutathione had beneficial effects on total motility, plasma membrane integrity, functionality and viability with lowering number of apoptotic spermatozoa. The difference in concentrations might be due to species, breed, extender differences or due to all.

It is well-known that cryopreservation prompts ROS production which affects the sperm functions[32]. Low scavenging and antioxidant power in sperm extender gave the chance for the generated ROS to drastically affect the sperm fertility characteristics like motility, viability, and penetration capability by interacting with its lipids, proteins and DNA[33]. The current study results were presented that addition of glutathione to Boer buck semen extender reduced aspartate-aminotransferase, alanine-aminotransferase and alkaline phosphatase enzyme concentrations, enhanced its total antioxidant capability, and reduced lipid peroxidation of the frozen-thawed semen; and this comes in harmony with Noei Razliqi *et al*[15] and Sarangi *et al*[31] who reported that glutathione had enhancing effects on semen characteristics. The improved post thawing motility in glutathione treated group (5 mM) might be elucidated by the intimate link between ROS, glutathione and motility. Glutathione efficiently combated ROS, so it not only guarded the sperm cells against the decrease in phosphorylation process of its axonemal proteins but also prevented membrane fluidity reduction[34], thus preventing the sperm immobilization. Plus, the reduction in glutathione concentration due to cryopreservation process resulted in changes in membrane transportation potential[9], which in turn affected its motility, penetration and fertilizing potential.

It is well established that the lipid peroxides generated by peroxidation during cryopreservation process can damage the sperm structure and eventually lower its metabolism and motility[35]. Lipid peroxides are unstable compounds; they decompose reactive carbonyl compounds, mainly MDA[36]. So MDA could be used as biochemical indicator for lipid peroxidation in spermatozoa[13]. The current study results reported that the presence of GSH in Boer buck semen extender reduced MDA level in frozen-thawed spermatozoa. These results came in agreement with Sikka[13] and Sarangi *et al*[31] who reported that glutathione addition to the semen extender reduced lipid peroxidation level in the sperm cell membrane. The end products of lipid peroxidation not only affected the sperm membrane and motility but also damaged its DNA integrity either through DNA bases oxidation or by binding with MDA[13,37], resulting in DNA deterioration, damage and an apoptotic-like changes[38–40], which appeared in the form of strand breaks and cross linking[13]. In harmony with these previous reports, the current study results showed that there were positive effects of GSH on Boer buck sperm freezability depending upon its roles in combating the oxidative hazardous of ROS on DNA, thus reducing its fragmentation[27,41]. Moreover, the current study results shed more light on the ideal concentration of GSH that should be incorporated in Boer buck semen. The present results confirmed that increased levels of glutathione inclusion in Boer buck semen extender had cytotoxic effects on the sperm functions. These results run in accordance with Aitken *et al*[42] who stated that high antioxidants levels in semen diluents accompanied with impaired sperm functions might be due to high sperm susceptibility to the cytotoxic effect of hydroperoxyl radical. Therefore, it is vital to select the ideal

antioxidant concentrations to maintain the natural balance between ROS production and scavenging activities[29].

In conclusion, this study confirms that the presence of GSH in Boer buck semen extender at a concentration of 5 mM improves its freezability. The protective glutathione effects against the cryo-injury are directly related to lipid peroxidation reduction and sperm DNA protection from deterioration by increasing total antioxidant capacity. Additionally, glutathione effect in Boer buck semen is dose-dependent, where 10 mM of glutathione is considered cytotoxic to Boer buck semen. Furthermore, the current study results open new windows to explore the practical application of antioxidants to improve the quality of post-thaw goat semen.

Conflict of interest statement

The authors declare that there was no conflict of interest.

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