INFLUENCE OF SUPPLEMENTING RAM SEMEN DILUENT WITH REDUCED GLUTATHIONE ON OXIDATIVE STRESS INDICES AND QUALITY ATTRIBUTES OF CHILLED AND CRYOPRESERVED SPERMATOZOA

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SUMMARY

This study was carried out to evaluate the efficiency of supplementing ram semen diluent with glutathione (GSH) on liquid-chilled and cryo-storage capacities of spermatozoa. Twenty ejaculates were collected from 5 Barki rams, 4 ejaculates each, during late-January and mid-February using an artificial vagina. The pooled specimens of each collection session were diluted (1:10) with Tris-citric egg yolk extender, and were split into 5 aliquots. The first aliquot served as control (GSH-free), whereas the other 4 aliquots were supplemented with 0.1, 0.2, 0.3 or 0.4 mMGSH. Thereafter, the samples were stored at 4°C for 3 h whereat each sample was subdivided into two portions. The first portion was maintained at 4°C for the subsequent 48 h during which sperm traits were assessed alongside oxidative stress indicators, whereas a chilled-glycerolized base diluent was added to the other portion and was further processed for cryopreservation. Post-thaw physical and kinematic properties of spermatozoa were evaluated by a computer-assisted sperm analysis (CASA) system, and sperm DNA fragmentation level within each group was also determined by fluorescent imaging. The results showed that GSH supplementation reduced (P<0.05) the oxidative stress on spermatozoa and maintained their physical and morphological criteria over the 48h period of chilled storage in a dose-depending pattern. Moreover, the 0.4 mM level of GSH improved (P<0.05) post-thaw traits and kinematics of ram sperm while decreasing (P<0.05) DNA fragmentation level compared to the control specimens. These findings imply glutathione's powerful potential as an exogenous antioxidant supplement in ram sperm medium which could be of great significance for further application in semen processing and IVF workflows.

Keywords: Glutathione, Ram, Semen, Cryopreservation, Oxidative stress

INTRODUCTION

Mammalian sperm are redox active cells that, spontaneously, generate reactive oxygen species (ROS) from respiration and metabolism of sperm cellular components. Ram sperm phospholipid cell membrane, in particular, contains substantial amounts of polyunsaturated fatty acids which renders it highly susceptible to oxidative damage (Van Tran *et al*., 2017). Excessive levels of ROS in sperm media initiate oxidative reactions that eventually lead to the death of the sperm cells (Guerra *et al*., 2004). Naturally, the seminal fluid contains an array of antioxidants that act as free radical scavengers to protect spermatozoa against oxidative stress (Soren *et al*., 2016). However, dilution of semen for processing deprives sperm cells from these essential, naturally-existed, antioxidants, which leads to sperm damage, deformity and eventually male infertility (Mostafa *et al*., 2009). Therefore, the application of antioxidants to semen diluents gained considerable attention in assisted reproductive technology and infertility practices (Hashem *et al*., 2017; and Rateb, 2018).

When semen is required within a short period of time after collection, chilled-diluted semen is considered a practical alternative to frozen semen to avoid freezing/thawing induced injuries (King *et al*., 2004). Nevertheless, improving semen quality during cold storage still appears to be a challenge in sheep industry (Hashem *et al*., 2017). In the meantime, the full potential of semen utilization relies on using it in the cryopreserved form (Ashrafi *et al*., 2013). However, cryopreservation exposes spermatozoa to an even extreme stress that leads to detrimental consequences on sperm and, thus, reduced post-thaw sperm traits (Meyers, 2005; Peña *et al*., and 2009; Rateb, 2018).

Over the past decades, several studies addressed this issue by applying different enzymatic and non-enzymatic antioxidants into semen diluents and evaluating their effects on reducing oxidative stress, hence enhancing chilled and cryopreserved ram sperm quality attributes as well as their fertilization competence (Zeitoun and Al-Damegh, 2015; Zaghloul *et al*., 2016; Khalifa, 2017a; Khalifa 2017b; Abd El-Hamid *et al*., 2018; Rateb, 2018; and Rateb *et al*., 2020). In continuation of this aspect, the current investigation aims to evaluate the capacity of supplementing ram semen diluent with reduced L-glutathione (GSH; γ-L-Glutamyl-L- cysteinylglycine) on neutralizing ROS-induced oxidative stress and, hence, enhancing sperm quality traits during chilled and cryo-storage. Sperm DNA fragmentation level was also visualized and assessed by fluorescent imaging as a direct indicator for sperm fertilization potential.

MATERIALS AND METHODS

Animals and management:

This study was conducted during late-January to mid-February using five mature Barki rams aged 36 - 48 months and an average body weight of 45.0±2.0 kg. The rams were kept in shaded pens belonging to

Artificial Insemination Lab., Mariout Research Station, Desert Research Center, Egypt. They were allowed for a daily grazing period from 0800 to 1400 h. Thereafter, they were provided a concentrate mixture to fulfill their protein and energy requirements (NRC, 2007), and Egyptian clover, *Trifolium alexandrinum*, hay *ad libitum*. Fresh water was available once daily after returning from the pasture. Prior to executing the experiment, all rams were clinically examined and were found free of disease or reproductive disorders. All experimental procedures described in the present work were conducted conforming to guidelines of the ethical committee for animal use of Desert Research Center.

Semen extender:

A Tris-citric acid egg yolk base extender was prepared 24 h prior to each collection session for dilution of ram semen as previously described (Rateb, 2018). Immediately after preparation, the diluent was centrifuged at 6000 rpm for 15 min and the clarified supernatant was collected. The clarified extender was stored at 4°C until use.

Semen collection :

Twenty ejaculates were collected from the rams, 4 ejaculates each, twice weekly throughout the period of study. Semen was collected by an artificial vagina as elaborated previously (Rateb *et al*., 2020). The ejaculates were maintained at 37°C during the collection sessions. Only ejaculates that exhibited acceptable sperm physical properties were processed.

Experimental design

The raw ejaculates were transported to the laboratory, in a water bath adjusted at 37°C, for further physical and cytological evaluation. Ejaculates from each collection session that exhibited a motility score equal or higher than four grade score (0= immotile, 5= highly-motile; David *et al.,* 2015) were pooled and diluted (1:10) with the base diluent. Thereafter, the diluted semen was split into 5 aliquots. The first aliquot (G1) served as control (glutathione-free), whereas each of the other four aliquots (G2-5) was supplemented with 0.1, 0.2, 0.3- or 0.4-mM L-glutathione reduced (Sigma-Aldrich, USA; Cat. no. G6013). Afterwards, all semen groups were stored at 4°C for 3 h whereat each sample was further subdivided into two portions. The first portion was maintained at 4°C throughout the subsequent 48 h, during which sperm traits were assessed alongside oxidative stress indicators at 24 h interval (T24 and T48). Meanwhile, a chilled-glycerolized base diluent was added to each of the other diluted portion to reach a final concentration of 2% glycerol (v/v) in medium. Thereafter, all glycerolized specimens were kept at 4°C for another 120 min before being packed in 0.25 mm French straws using a mini-tübe filling and sealing machine (Model 133, Mini-tübe, Germany). The straws were then placed horizontally in a mini-tübe biological freezer and were exposed to nitrogen vapor (-80°C) for 10 min. The frozen straws were eventually immersed and stored in liquid nitrogen (-196°C) until analyses.

Determining oxidative stress indicators and enzymatic activities during 48h of chilled storage*:*

A 2 ml portion of each chilled semen group was obtained and centrifuged (2500 rpm for 10 min) at times parallel to those of sperm physical and morphological assessment (T0, T24 and T48). The supernatant was collected and stored at -20 oC until oxidative stress indicators and enzymatic activities were analyzed. The changes in malondialdehyde acetate (MDA) concentration, total antioxidant capacity (TAC) and alkaline phosphatase (ALP) activity in medium were analyzed by colorimetric kits (Biodiagnostic, Egypt). Moreover, activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed colorimetrically by kits obtained from Spectrum, Egypt. All procedures were carried out following instructions provided by the manufacturers.

Semen assessment:

Sperm progressive motility (%) was evaluated in 5 random fields using a phase-contrast microscope (Leica) at 40 X magnification. Sperm vitality (%) was assessed under high power magnification (100×) after mixing and smearing 10 µl of semen and 5 µl of freshly-prepared eosin-nigrosin stain on a warm glass slide. Sperm abnormalities and acrosome integrity were evaluated using Romanowski's triple-stain technique (DIFF-QUICK III, Vertex, Egypt), and stained smears were evaluated by a phase-contrast microscope at 1000x magnification. Sperm plasma membrane integrity was determined by the hypo-osmotic swelling test (HOST) according to the method of Mosaferi *et al*. (2005), where at least 200 sperm were evaluated at 40 X magnification.

Computer-assisted semen analysis (CASA):

The frozen straws (five per treatment) were thawed in a programmable thawing device (mini-tube, Germany) adjusted at 38°C for 40 seconds. Promptly, each sample was evaluated for sperm physical and kinematic traits by a computer-assisted semen analysis (CASA) system (Mira-9000, Mira Lab, Egypt) following the world health organization strict criteria (WHO, 2010). Prior to assessment, the system was calibrated for normal ram sperm motility and morphometric properties. Ten random fields were assessed at 500x magnification, where a minimum of 200 sperm were evaluated for motility parameters; i.e., total motility (%), progressive motility (%), non-progressive motility (%), proportion of immotile sperm (%) and viability (%). Sperm kinematics criteria; i.e., straight line velocity (VSL, µm/s), curvilinear velocity (VCL, µm/s), path velocity (VAP, µm/s), amplitude of lateral head displacement (ALH, µm), wobble movement (WOB, %), linearity (LIN, %) and straightness (STR, %) were also recorded.

Sperm DNA fragmentation assessment:

The level of sperm DNA fragmentation in control and glutathione-treated groups was determined by kits obtained from Halomax® (Halotech® DNA SL Corp., Madrid, Spain) as previously elaborated in other species (Rateb, 2021). Sperm DNA fragmentation index (DFI, %) was calculated as the proportion of DNA-fragmented spermatozoa of total sperm count (Yang *et al*., 2019).

Statistical analysis:

The normal distribution of data was checked using the Shapiro-Wilk test and when the distribution was not normal data were logged to improve the approximation of normality. The changes in sperm physical and morphological characteristics, as well as oxidative stress indicators and enzymatic activities, throughout the 48h period of chilled storage were analyzed by repeated measures analysis of variance (ANOVA) to determine the fixed effects of treatment, time (T0, T24 and T48) and treatment by time interaction. The same procedure was used to determine the changes in sperm traits of cryopreserved semen, where the fixed effects of treatment level, time (T0, T5 and Tpost-thaw) and level by time interaction were obtained. The differences between means were detected by Duncan's post-hoc test at 5% significance, and data were analyzed using IBM-SPSS statistics program (IBM-SPSS, 2013). The data are expressed as means ±standard error (SEM).

RESULTS

Effects on sperm quality criteria during 48h of chilled storage:

The results showed that sperm physical and morphological criteria were affected (P<0.05) by glutathione supplementation, in a dose-depending pattern, over time of cold storage. In this regard, both higher levels of glutathione (i.e., 0.3 and 0.4 mM) recorded the highest (P<0.05) sperm motility over the 48h of preservation (T24 and T48) compared to the other semen groups (table 1). Likewise, the same higher glutathione level groups recorded the highest (P<0.05) percentages of live and normal spermatozoa over time of chilled storage (table 1). No significant difference was observed for glutathione supplementation on the percent of primary sperm abnormalities. However, the highest (P<0.05) values of secondary sperm abnormalities were recorded in both control and low-level glutathione supplementation compared to all other supplemented specimens (table 1). On the other hand, the higher levels of glutathione supplementation corresponded with highest (P<0.05) values of intact acrosome and sperm cell membrane integrity over the period of cold storage (table 1).

Table 1. Effect of different levels of glutathione supplementation on physical traits of ram semen during 48 h of chilled storage at 4 ºC (Mean ±SEM)

Parameter	Time (h)	Level of glutathione in medium				
		Control	G_1 0.1 mM	G_2 0.2 mM	G_3 0.3 mM	G_4 0.4 mM
Progressive Motility (%)	0	90.0 ^{A} ±2.2	88.0 ^{A} ±1.2	91.0 ^{A} ±2.4	94.0 ^{A} ±1.0	94.0 ^{A} ±1.0
	24	70.0 $^{c, B}$ ±1.6	78.0 $^{b, B}$ ±2.0	79.0 $^{b, B}$ ±3.7	83.0 $^{ab, B}$ ±1.2	86.0 $^{a, B}$ ±1.0
	48	49.0 $^{c, C}$ ±4.5	63.0 $^{b, C}$ ±2.0	68.0 $^{b, C}$ ±3.7	77.0 $^{a, C}$ ±1.2	82.0 $^{a, C}$ ±1.2
Live sperm (%)	0	91.4 ^{A} ±0.7	91.4 ^{A} ±0.4	93.6 ^{A} ±3.1	91.4 ^{A} ±2.2	93.6 ^{A} ±0.9
	24	72.6 $^{b, B}$ ±1.1	74.0 $^{b, B}$ ±1.0	79.6 $^{ab, B}$ ±3.9	77.0 $^{ab, B}$ ±3.7	86.4 $^{a, B}$ ±0.2
	48	61.4 $^{b, C}$ ±0.6	61.6 $^{b, C}$ ±0.4	76.2 $^{a, B}$ ±6.6	78.0 $^{a, B}$ ±2.4	84.8 $^{a, B}$ ±0.5
Normal sperm (%)	0	84.8 ^{A} ±0.5	84.4 ^{A} ±0.4	87.8 ^{A} ±2.2	85.4 ^{A} ±1.5	87.4 ^{A} ±1.5
	24	70.8 $^{b, B}$ ±0.5	71.6 $^{b, B}$ ±0.4	79.6 $^{a, B}$ ±3.9	77.0 $^{a, B}$ ±1.2	83.2 $^{a, AB}$ ±1.9
	48	66.8 $^{b, C}$ ±0.7	65.6 $^{b, C}$ ±0.6	73.2 $^{a, B}$ ±2.9	76.4 $^{a, B}$ ±3.2	76.2 $^{a, B}$ ±1.9
Primary abnormalities (%)	0	2.2 ±0.3	2.8 ±0.2	1.4 ±0.4	2.6 ±0.2	2.0 ±0.1
	24	2.4 ±0.4	2.0 ±0.1	2.6 ±0.2	1.4 ±0.2	2.4 ±0.2
	48	2.4 ±0.4	2.0 ±0.1	2.6 ±0.2	1.4 ±0.2	2.4 ±0.2
Secondary abnormalities (%)	0	13.0 ^{C} ±0.5	12.8 ^{C} ±0.2	10.8 ^{C} ±2.1	12.0 ^{B} ±1.2	10.6 ^{B} ±1.5
	24	26.8 $^{a, B}$ ±0.5	26.4 $^{a, B}$ ±0.4	17.8 $^{bc, B}$ ±4.2	21.6 $^{ab, A}$ ±1.5	14.4 $^{c, B}$ ±2.2
	48	30.8 $^{a, A}$ ±0.9	32.4 $^{a, A}$ ±0.4	24.2 $^{bc, A}$ ±3.1	22.2 $^{c, A}$ ±3.1	21.4 $^{c, A}$ ±2.2
Intact acrosome (%)	0	86.8 ^{A} ±1.4	88.8 ^{A} ±1.2	86.4 ^{A} ±6.0	90.4 ^{A} ±5.4	91.0 ^{A} ±0.2
	24	67.0 $^{c, B}$ ±1.2	66.0 $^{bc, B}$ ±1.0	80.2 $^{a, B}$ ±4.2	73.8 $^{ab, B}$ ±5.3	87.4 $^{a, A}$ ±0.2
	48	33.2 $^{b, C}$ ±0.7	32.8 $^{b, C}$ ±0.9	44.2 $^{b, C}$ ±6.0	49.6 $^{b, C}$ ±8.3	74.8 $^{a, B}$ ±0.9
Intact cell membrane (%)	0	88.6 ^{A} ±0.4	83.6 ^{A} ±2.2	84.6 ^{A} ±2.0	88.8 ^{A} ±2.0	90.6 ^{A} ±0.9
	24	63.6 $^{b, B}$ ±1.8	61.6 $^{b, B}$ ±1.6	71.7 $^{ab, B}$ ±3.6	72.6 $^{a, B}$ ±4.4	75.0 $^{a, B}$ ±0.9
	48	27.0 $^{c, C}$ ±2.0	23.6 $^{c, C}$ ±1.6	42.0 $^{b, C}$ ±2.0	43.2 $^{b, C}$ ±5.8	65.4 $^{a, C}$ ±1.5

$^{a-c}$ letters among groups in the same row differ significantly (P < 0.05), $^{A-C}$ letters in the same column within each parameter differ significantly (P < 0.05), Intact cell membrane, HOST-reacted spermatozoa.

Effects on oxidative stress indices and enzymatic activities:

In the meantime, the highest (P<0.05) level of total antioxidant capacity (TAC) was recorded in the specimens treated with the highest glutathione supplementation (0.4 mM) at 48h of preservation time compared to control and other treated groups (table 2). However, both higher GSH-treated groups recorded the lowest (P<0.05) malondialdehyde acetate (MDA) levels compared to control as well to the other treated groups throughout the 48h of chilled storage (table 2).

Furthermore, the control group recorded the highest (P<0.05) ALT activity at 48h of chilled preservation, while the lowest (P<0.05) activity was recorded at T0. Nevertheless, no significant difference was observed in ALT activity among all glutathione-treated groups over the 48h preservation period (table 2). On the other hand, the lowest (P<0.05) levels of aspartate aminotransferase (AST) and alkaline phosphatase (ALP) was recorded in all groups at T0, whereas the highest (P<0.05) levels were observed at T0 of preservation. Worthwhile, specimens supplemented with high glutathione concentration (0.3, 0.4 mM) recorded the lowest (P<0.05) activities for both AST and ALP throughout the 48h of chilled storage compared to all other groups (table 2).

Table 2. Effect of incorporating glutathione into ram sperm diluent on oxidative status and enzymatic activities during 48 h of chilled storage at 4ºC (Mean± SEM)

Parameter	Time (h)	Level of glutathione in medium				
		Control	G_1 0.1 mM	G_2 0.2 mM	G_3 0.3 mM	G_4 0.4 mM
TAC (mM/L)	0	0.76^{A} ±0.05	0.76 ±0.03	0.66 ±0.04	0.59 ±0.04	0.54^{C} ±0.03
	24	0.56^{B} ±0.09	0.68 ±0.09	0.56 ±0.02	0.55 ±0.06	0.66^{B} ±0.07
	48	$0.51^{c, B}$ ±0.02	0.72^{b} ±0.05	0.51^{b} ±0.05	0.60^{b} ±0.05	$0.83^{a, A}$ ±0.05
MDA (nM/L)	0	10.4^{B} ±1.4	10.8^{B} ±0.5	10.6^{B} ±0.8	9.2 ±1.0	9.0 ±1.6
	24	$20.1^{a, A}$ ±1.2	$12.0^{b, A}$ ±0.3	$10.1^{b, B}$ ±0.7	9.3^{bc} ±1.0	9.2^{c} ±1.1
	48	$26.7^{a, A}$ ±1.9	$12.3^{b, A}$ ±1.9	$12.1^{a, A}$ ±1.9	9.3^{c} ±1.9	9.3^{c} ±1.9
ALT (U/L)	0	75.9^{B} ±3.9	77.3 ±2.5	73.5 ±2.1	75.5 ±2.2	70.9 ±3.4
	24	$104.0^{a, A}$ ±2.4	71.4^{b} ±1.6	76.1^{b} ±1.2	74.5^{b} ±2.7	72.0^{b} ±3.0
	48	$105.5^{a, A}$ ±3.5	77.6^{b} ±3.6	77.2^{b} ±3.6	77.2^{b} ±3.6	76.2^{b} ±3.6
AST (U/L)	0	$225.3^{a, C}$ ±4.6	$210.8^{a, C}$ ±4.9	$194.5^{b, C}$ ±5.5	195.2^{b} ±5.2	196.5^{b} ±3.4
	24	$231.6^{a, B}$ ±8.3	$221.0^{a, B}$ ±4.1	$210.0^{a, B}$ ±3.9	196.5^{b} ±6.5	192.8^{b} ±3.0
	48	$240.1^{a, A}$ ±7.2	$238.5^{a, A}$ ±5.3	$238.3^{a, A}$ ±5.3	196.2^{b} ±5.3	192.0^{b} ±5.3
ALP (IU/L)	0	$253.1^{a, C}$ ±5.3	$224.9^{a, C}$ ±6.9	$208.4^{ab, C}$ ±6.0	$217.5^{b, B}$ ±8.6	$199.4^{c, B}$ ±6.9
	24	$255.3^{a, B}$ ±5.0	$234.0^{ab, B}$ ±6.9	$220.0^{bc, B}$ ±6.6	$212.5^{c, A}$ ±9.4	$182.5^{d, B}$ ±6.3
	48	$264.0^{a, A}$ ±7.8	$236.3^{ab, A}$ ±7.6	$251.1^{ab, A}$ ±7.6	$215.4^{b, A}$ ±7.6	$174.1^{c, A}$ ±7.6

$^{a-d}$ Values in the same row with different superscript letters differ significantly (P< 0.05), ^{A,B} Values in the same column with different superscript letters differ significantly (P< 0.05), TAC, total antioxidant capacity; MDA, malondialdehyde acetate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase.

Effects on post-thaw sperm physical, morphometric and kinematic properties:

The CASA-derived results of the present study showed that, except for the percent of primary abnormalities, glutathione supplementation profoundly affected (P<0.05) post-thaw physical and cytological properties of cryopreserved ram spermatozoa (table 3). In that regard, the lowest (P<0.05) percentages of progressive motility, viability, normal sperm, intact acrosome and intact sperm cell membrane were recorded in the control group post thawing compared to all glutathione-supplemented groups (table 3). This was accompanied by recording the highest (P<0.05) percent of sperm secondary abnormalities in the control group compared to all glutathione-treated specimens (table 3). Meanwhile, the lowest (P<0.05) percent of post-thaw sperm secondary abnormalities were observed in the groups supplemented with high glutathione levels backed by the highest (P<0.05) percent of progressive motility, viability, normal sperm, intact acrosome and intact sperm cell membrane post thawing (table 3).

Regarding post-thaw kinematics of spermatozoa, the control group recorded the highest (P<0.05) percent of immotile sperm, while recording the lowest (P<0.05) progressive motility, curvilinear velocity (VCL), straight-line velocity (VSL), Average path velocity (VAP) and amplitude of lateral head displacement (ALH) compared to glutathione-treated groups (table 4).

Table 3. Effect of different levels of glutathione supplementation on physical properties of cryopreserved ram spermatozoa (Mean ±SEM)

Parameter	Time (h)	Level of glutathione in medium				
		Control	G_1 0.1 mM	G_2 0.2 mM	G_3 0.3 mM	G_4 0.4 mM
Progressive Motility (%)	T_0	91.0 ^{A} ±1.8	90.0 ^{A} ±2.2	93.0 ^{A} ±1.2	94.0 ^{A} ±1.0	94.0 ^{A} ±1.9
	T_5	85.0 ^{A} ±3.1	84.0 ^{A} ±1.9	84.0 ^{B} ±1.9	88.0 ^{A} ±1.2	92.0 ^{A} ±2.0
	Post-thaw	54.0 $^{b, B}$ ±1.2	72.7 $^{ab, B}$ ±1.4	72.9 $^{ab, C}$ ±0.9	75.1 $^{a, B}$ ±1.3	79.7 $^{a, B}$ ±2.4
Live sperm (%)	T_0	82.8 ^{A} ±1.7	87.2 ±2.9	86.4 ±2.6	85.2 ±1.3	88.4 ±3.6
	T_5	82.0 ^{A} ±1.6	85.0 ±1.0	84.5 ±1.4	85.0 ±1.7	85.8 ±1.9
	Post-thaw	51.4 ±1.6 $^{b, B}$	83.2 ^{a} ±1.7	83.4 ^{a} ±1.0	84.0 ^{a} ±1.4	85.2 ^{a} ±1.5
Normal sperm (%)	T_0	85.8 ^{A} ±1.2	88.4 ^{A} ±0.2	87.8 ^{A} ±0.9	88.0 ^{A} ±1.0	87.0 ±1.1
	T_5	81.2 ^{A} ±1.4	86.2 ^{A} ±0.5	84.6 ^{A} ±0.9	84.2 ^{A} ±0.8	86.4 ±2.2
	Post-thaw	47.0 $^{c, B}$ ±2.5	76.0 $^{b, B}$ ±1.6	78.5 $^{ab, B}$ ±0.5	79.0 $^{ab, B}$ ±1.0	83.5 ^{a} ±1.6
Primary abnormalities (%)	T_0	2.6 ±0.5	1.8 ±0.2	2.4 ±0.4	2.0 ±0.5	2.0 ±0.4
	T_5	2.5 ±0.4	2.0 ±0.4	2.6 ±0.2	2.8 ±0.5	2.0 ±0.2
	Post-thaw	2.6 ±0.4	2.0 ±0.4	2.6 ±0.2	2.8 ±0.2	2.8 ±0.4
Secondary abnormalities (%)	T_0	11.6 ^{C} ±1.1	9.8 ^{C} ±1.2	9.8 ^{B} ±1.4	10.0 ^{C} ±1.4	11.0 ±1.5
	T_5	16.3 ^{B} ±1.7	11.8 ^{B} ±1.4	17.8 ^{A} ±1.2	13.0 ^{B} ±1.5	11.6 ±1.9
	Post-thaw	50.4 $^{a, A}$ ±2.1	22.0 $^{b, A}$ ±1.4	12.8 $^{bc, A}$ ±1.1	18.2 $^{bc, A}$ ±1.1	13.7 ^{c} ±2.0
Intact acrosome (%)	T_0	88.0 ^{A} ±1.7	89.8 ^{A} ±1.5	90.4 ^{A} ±2.4	90.8 ^{A} ±1.2	92.4 ^{A} ±1.2
	T_5	79.4 ^{B} ±1.2	86.2 ^{B} ±1.5	86.2 ^{B} ±2.8	85.8 ^{B} ±1.8	87.0 ^{A} ±1.8
	Post-thaw	42.8 $^{c, C}$ ±2.3	78.0 $^{b, C}$ ±2.9	78.0 $^{b, C}$ ±2.6	80.4 $^{a, B}$ ±1.6	82.4 $^{a, B}$ ±1.9
Intact cell membrane (%)	T_0	87.8 ^{A} ±0.7	85.4 ^{A} ±1.5	88.0 ^{A} ±1.3	89.2 ^{A} ±0.2	91.2 ^{A} ±1.2
	T_5	79.6 ^{B} ±0.8	78.0 ^{B} ±1.7	84.4 ^{B} ±1.6	84.5 ^{B} ±0.6	84.5 ^{B} ±1.6
	Post-thaw	39.4 $^{c, C}$ ±0.9	72.7 $^{b, B}$ ±2.8	73.2 $^{ab, B}$ ±1.5	81.0 $^{b, B}$ ±0.9	83.0 $^{a, B}$ ±1.1

$^{a-c}$ letters among groups in the same row differ significantly ($P < 0.05$), $^{A-C}$ letters in the same column within each parameter differ significantly ($P < 0.05$), **T_0,** Immediately after dilution; **T_5,** after 5 h equilibration at 4^{o}C (prior to cryopreservation).

Table 4. Mean values of post-thaw kinematics of cryopreserved ram spermatozoa following supplementation with different levels of glutathione (Mean ±SEM)

Parameter	Level of glutathione in the diluent				
	Control	G_1 0.1 mM	G_2 0.2 mM	G_3 0.3 mM	G_4 0.4 mM
Prog. Motility (%)	26.0 ^{c} ±1.3	35.1 ^{b} ±1.1	35.3 ^{b} ±1.5	36.9 ^{b} ±1.2	41.6 ^{a} ±1.9
Non-Prog Mot. (%)	33.3 ±1.8	33.1 ±1.5	33.9 ±1.3	32.6 ±1.1	33.2 ±1.3
Immotile (%)	40.7 ^{a} ±1.1	31.8 ^{b} ±1.6	30.8 ^{b} ±1.1	30.5 ^{b} ±1.2	25.2 ^{c} ±1.5
VCL (µm/s)	32.0 ^{b} ±3.1	45.8 ^{a} ±2.9	53.4 ^{a} ±3.6	48.3 ^{a} ±3.9	44.0 ^{a} ±3.8
VSL (µm/s)	11.8 ^{c} ±2.7	21.2 ^{b} ±2.6	21.9 ^{b} ±2.0	25.5 ^{ab} ±2.2	28.7 ^{a} ±2.8
VAP (µm/s)	16.1 ^{b} ±1.6	25.9 ^{a} ±3.0	20.8 ^{a} ±1.3	27.5 ^{a} ±2.0	23.2 ^{a} ±3.1
ALH (µm)	1.8 ^{b} ±0.4	3.6 ^{a} ±0.3	3.2 ^{a} ±0.5	3.4 ^{a} ±0.3	3.1 ^{a} ±0.3
LIN (%)	40.0 ±1.7	44.4 ±1.2	46.4 ±1.7	42.9 ±2.2	42.4 ±1.6
WOB (%)	52.9 ±1.8	55.8 ±1.7	57.1 ±1.7	55.7 ±1.6	53.2 ±1.7
STR (%)	72.6 ±1.6	77.3 ±1.9	78.9 ±1.3	73.4 ±1.8	77.9 ±1.9

$^{a-c}$ letters within each parameter differ significantly ($P < 0.05$), **Prog. Motility**, Progressive motility, **Non-Prog Mot**, Non-progressive motility, VCL, Curvilinear velocity, **VSL**, Straight-line velocity, **VAP**, Average path velocity; **ALH**, amplitude of lateral head displacement, **WOB**, Wobble movement coefficient, **LIN**, Linearity; **STR**, Straightness.

Effects on sperm DNA integrity:

Post-thaw fluorescent evaluation of sperm nucleoids showed that the magnitude of DNA fragmentation in all semen specimens was affected ($P<0.05$) by cryopreservation of semen. In this regard, the highest ($P<0.05$) post-thaw sperm DNA fragmentation index (DFI) was observed in the control group (75.0 ±1.9 %), whereas the lowest ($P<0.05$) was recorded in the groups supplemented with high levels of GSH with values 37.5 ±0.8 and 31.9 ±0.9 %, respectively (Figure 1).

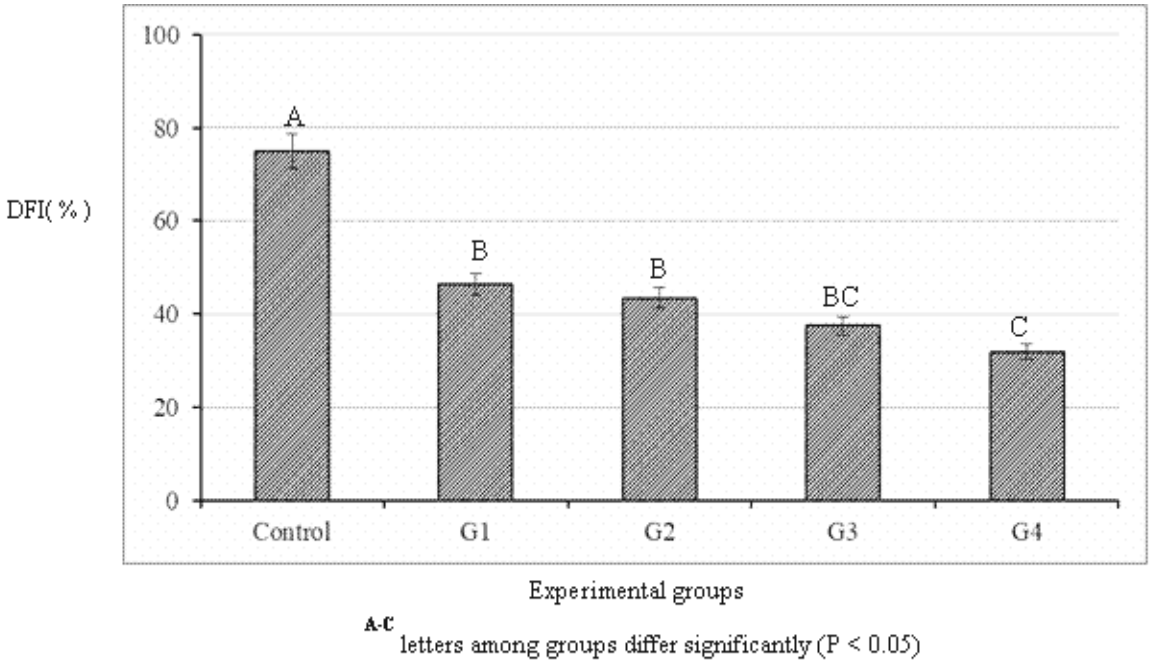

Fig 1. Effect of different levels of glutathione supplementation into sperm medium on post-thaw sperm DNA fragmentation index (DFI, %) (mean ±SEM), **Control,** glutathione-free; **G1 through G4,** specimens were supplemented with 0.1, 0.2, 0.3 and 0.4 mM glutathione, respectively, prior to processing for cryopreservation.

DISCUSSION

This work aimed to improve chilled storage and cryopreservation capacities of ram sperm by adding reduced L-glutathione (GSH) to the extender. The results showed that supplementing sperm preservation medium with 0.4 mMGSH efficiently compensated for the typical decrease in GSH content occurring during sperm processing and mitigated the drastic effects of oxidative stress, hence enhancing quality attribute of chilled and cryo-stored spermatozoa. This was clearly evident since that the 0.4 mMGSH-supplemented group recorded percentages of sperm progressive motility, viability, intact acrosome and intact cell membrane 1.7, 1.4, 2.3 and 2.4 times higher than those of the control group, respectively. This was accompanied by higher values of TAC and lower levels of MDA production and other enzymatic activities over the 48h chilled storage period. Even further, the potency of GSH supplementation on mitigating the drastic effects of oxidative stress even extended to cryopreservation of spermatozoa since the same GSH-treated group recorded 1.5, 1.7, 1.9 and 2.1 times improvement in post-thaw sperm motility, viability, intact acrosome and HOST-reacted spermatozoa over those of control, respectively.

Typically during freezing, two important incidents occur; i.e production of ROS that induce changes in sperm membrane function and structure (Ball *et al*., 2001), and alteration in sperm antioxidant defense systems including a decrease in intracellular GSH content (Gadea *et al*., 2004; Gadea *et al*., 2005a; and Gadea *et al*., 2005b). Therefore, it is suggested that supplementing sperm preservation medium with GSH to compensate for such occurring reduction efficiently protected ram spermatozoa from oxidative damage, hence improved sperm viability and functional integrity observed in this work during liquid-chilled storage or cryopreservation. This is in agreement with previous reports in other species (Bilodeau *et al*., 2001; Foote *et al*., 2002; and Gadea *et al*., 2008)

Glutahione (γ-L-Glutamyl-L- cysteinylglycine) is a tripeptide comprised of one molecule of L-glutamic acid, L-cysteine, and Glycine each. It is ubiquitously distributed in living cells and plays a pivotal role as an intracellular defense mechanism against oxidative stress. In context, GSH is an important regulator of the scavenging system and one of the most important nonenzymatic antioxidants in sperm cells (Gadea *et al*., 2008). This would explain the decrease in MDA generation as well as other enzymatic activities in the GSH-supplemented groups in the present study, and would indicate that glutathione supplementation was responsible, at least in part, for the lower disruption of sperm cell lipid packing.

On the other side, chromatin condensation and stability are critical factors to consider when using frozen semen for AI or IVF programs (Madrid-Bury *et al*., 2005). In the regard, GSH has been reported to have a direct decondensation effect on sperm chromatin (Delgado *et al*., 2001). Interaction of sperm chromatin with ROS accumulated in the medium may lead to oxidizing the sulphydryl group and increase disulfide bond cross linking and higher chromatin compactness. This would explain the lower DNA damage observed in the GSH-supplemented groups compared to that of control in

the present work. Additionally, the dose-depending pattern observed in response to different GSH supplementation levels in the medium would also reflect an adequate redox balance (i.e. balance between ROS generation in medium and level of GSH supplementation), hence less disruption of the condensation and stability of sperm chromatin after cryopreservation (Gadea *et al*., 2008).

CONCLUSION

The results obtained in this study concluded that addition of GSH to ram semen extender efficiently prevented occurrence of redox imbalance in the preservation medium, hence improving physical and functional attributes of both chilled-stored and cryopreserved spermatozoa. Further more, the results reflect GSH's powerful potential to protect sperm DNA from redox-related damage, which would be a significant benefit when manipulating semen for artificial insemination (AI) and in-vitro fertilization (IVF) schemes.

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تأثير دعم مخفف السائل المنوي للكباش بالجلوتاثيون على مؤشرات اجهاد الأكسدة وخصائص الحيوانات المنوية المحفوظة بالتبريد والتجميد

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أجريت هذه الدراسة بهدف تقييم كفاءة دعم بيئة تداول السائل المنوي للكباش بالجلوتاثيون في الحفاظ على المقدرة الحفظية للحيوانات المنوية سواء في صورة سائلة مبردة أو في صورة مجمدة. تم جمع ٢٠ قذفة سائل منوي من خمسة كباش برقي، أربعة من كل كبش، بواسطة مهبل اصطناعي خلال الفترة من منتصف يناير حتى منتصف فبراير. تم دمج القذفات عالية الجودة من الكباش ثم تم تخفيفها (١:١٠) بمخفف الترس-سيتريك صفار البيض ثم قسمت القذفات المخففة الى خمس مجموعات تجريبية. المجموعة الأولى مثلت مجموعة المقارنة (خالية من الجلوتاثيون)، بينما دعمت المجموعات الاربع الاخرى بتركيزات ٠,١ و٠,٢ و٠,٣ و٠,٤ ملي مول من الجلوتاثيون على التوالي، ثم وضعت جميع المعاملات في ثلاجة (4º م) لمدة ثلاث ساعات وعندها قسمت عينات كل معاملة الى جزئين. الجزء الأول تم استمرار حفظه في صورة سائلة مبردة كما هو (4º م) لمدة ٤٨ ساعة تم خلالها تقييم الخصائص الطبيعية والخلوية للحيوانات المنوية جنباً الى جنب مع مؤشرات اجهاد الأكسدة، في حين أضيف الى الجزء الآخر مخفف مدعم بالجليسرول (تركيز نهائي في العينة ٢٪) توطئة لتداولها وحفظها في صورة مجمدة. تم تقييم الخصائص الطبيعية والحركية للحيوانات المنوية قبل وبعد التجميد والاسالة بواسطة جهاز CASA، كما تم تقدير معدلات تلف الحامض النووي الديؤكسي ريبوزي DNA للحيوانات المنوية بواسطة التصوير الفلوريسينتي كمؤشر مباشر على المقدرة الإخصابية للحيوانات المنوية. وقد أظهرت النتائج أن دعم بيئة تداول وحفظ الحيوانات المنوية بالجلوتاثيون قلل معنوياً ($P<0.05$) من إجهاد الأكسدة على الحيوانات المنوية كما حافظ على خصائصها الطبيعية والمورفولوجية طوال فترة الحفظ (٤٨ ساعة) في صورة سائلة مبردة. كما اوضحت النتائج أن دعم بيئة تداول وحفظ الحيوانات المنوية بـ ٠,٤ ملي مول من الجلوتاثيون قد حسن معنوياً ($P<0.05$) من الخصائص الحيوية والحركية للحيوانات المنوية، كما أظهر أقل ($P<0.05$) نسبة تلف للـ DNA في الحيوانات المنوية بعد التجميد والإسالة بما يعكس الحفاظ على مقدرتها الإخصابية. تشير هذه النتائج الى قوة وكفاءة الجلوتاثيون كمضاد للأكسدة حال استخدامه في دعم بيئات تداول وحفظ الحيوانات المنوية للكباش والتي يمكن استخدامها مستقبلاً في برامج الإخصاب المجهري.