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THE EFFECTS OF INCLUDING GLUTATHIONE ON THE CHARACTERISTICS OF POST-THAWED SEMEN IN ARABIAN HORSE

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ABSTRACT

This research aimed at investigating the effects of adding glutathione as an antioxidant to the Arabian horse semen extender on the sperm viability and integrity after cooling and freezing. Glutathione was tested at 0.5, 1, 2, and 4 mM/100 mL in the basic extender (HF-20). HF-20 extender was used as the basic control extender. Adding glutathione at a concentration of 2mM showed a high increase in the total motility sperm (61.25 ± 3.14%), progressive motility (28.13 ± 2.07 %),and VCL (90.03 ± 1.18µm/s)compared to other concentrations and to the control group. As well, it was significantly higher in normal morphology (80.07 ± 1.30 %) compared with 4 mM of glutathione. However, the increase in the glutathione values resulted in less integrity of the acrosome and HOST. There was no significant difference in sperm vitality among glutathione groups and control group. In conclusion, improvement of motility, and speed parameters by supplementing arginine at 2mM, but integrity of acrosome at 0.5 mM to freeze-extender horse semen. In addition, we urge conducting more research into adding this substance and performing artificial insemination in mare.

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INTRODUCTION

Domestication of the Arabian horse in the Arabian Peninsula has been dated back to 9000 years ago (Schiettecatte and Zouache, 2017). Raising Arabian horses in the Arabian Gulf region is of great importance for the people inhabiting this area of the globe, due to not only the economic aspects but also represents the heritage value for the society. Events for competitions of horse racing and beauty parades are regularly held annually in this region of the world. In Islamic culture and history, the horse constitutes a source of wealth, endurance, and military power. With the progress of molecular techniques, several studies (Ropka-Molik*et al.,* 2019; Cosgrove *et al.,* 2020) have investigated the mitochondrial DNA, proteomics, and metabolomics in the Arabian horses to identify the genes responsible for the endurance and adaptation for arid climates. To propagate the population of horses, some advancement must be exercised such as the implementation of semen collection and artificial insemination. There appears to exist a genetic factor affecting stallion semen cryopreservation. Optimizing a proper diluent for Arabian horse semen necessitates many efforts on the type and levels of the extender components. Several antioxidants and nutrients have been supplemented and tried to the buffer-based extenders. Glutathione has been supplemented to several species of semen and resulted in improvements in the case of cooling (Ahmed *et al.,* 2021) and freezing (Ogata *et al.,* 2015). During semen processing and storage steps, there produced free radicals due to the active metabolic mechanism of the sperm cells at ambient temperature. This reaction results in increased levels of ROS (i.e. mainly H2O2 and NO2-) which damage the integrity of the sperm cells, resulting in low fertilization. Types of antioxidants are divided into two categories; enzymatic and non-enzymatic factors. Of the enzymatic sources as antioxidants are superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), ά dioxygenase, ascorbate peroxidase, dehydroascorbatereductases, glutathione reductase, glutathione-S-transferase, and NADPH oxidase (Irato and Santovito, 2021). The same authors stated that the non-enzymatic agents are ascorbate, fibrin, glutathione, melatonin, mycothiol, phenolics, vitamin E, cysteine, and serum albumin. Glutathione, however, was found to be a potent antioxidant that improves sperm characteristics in rabbits (Ahmed *et al*., 2021), dogs (Ogata *et al.,* 2015), bulls (Shah *et al.,* 2017), avian (Partyka and Nizaunski, 2021), and horses (Del Prete*et al*., 2019). Therefore, the aims of the study way to evaluate the role of glutathione as an antioxidant supplemented to the basic extender (HF-20) on the post-thaw sperm characteristics of Arabian horses which are native to the Arabian Peninsula.

MATERIALS AND METHODS

Animals: Five healthy stallions aged 4 to 10 years were selected for this study after a breeding soundness examination. Animals were housed individually in a private stud and offered pellets, alfalfa hay, clean water, and integrated mineral licks.

Semen collection: Estrous-mare was used for semen collection in this trial. After semen collection, the gel was removed immediately from the semen. The sample was collected using sterile gauze and transferred to a water bath at 37°C. The semen volume was measured in a graduated cylinder. The ejaculate was then evaluated for initial progressive motility and sperm concentration. Sperm concentration and motility were determined using the CASA system (ISAS program, Prosser R+D, Paterna, Valencia, Spain). Samples with a minimum concentration of 200×10^6 sperm/ml and motility > 60% were used in the study.

Freezing Protocol: The filtered semen of each ejaculate was diluted (1:1) with a centrifuged medium and then divided into six aliquots. The aliquots were centrifuged at 900 g for 10 minutes, the seminal plasma was removed, and each sample was re-suspended with HF-20 (basic extender) without any additives and served as a control. HF-20 extender was supplemented with antioxidants glutathione at 0.5, 1, 2, and 4 mM. The final semen concentration after dilution was 200×10^6 sperm/ml. All of the tubes were cooled to 4°C within 90 minutes before being assessed for motility, morphology, and sperm membrane integrity. Cooled semen was filled into 0.5 ml straws. The straws were frozen in a horizontal position on the liquid nitrogen surface (9 cm above the L-N2 level) for 9 minutes.

Extenders and media: The centrifuged medium was a mixture of 6.0 g glucose, 0.37 g ethylenediamine tetra-acetic acid (EDTA), 0.37 g sodium citrate, 0.12 g sodium bicarbonate, 100,000 IU penicillin, and 0.08 g streptomycin in 100 ml of distilled water. Freezing extender with egg yolk was prepared by dissolving 5.0 g glucose, 0.3 g lactose, 0.3 g raffinose, 0.15 g sodium citrate, 0.05 g sodium phosphate, 0.05 g sodium potassium tartrate, and 3 mL glycerol in 50 mL distilled water. Added to this buffer a 10 mL EY and distilled water was added up to a total volume of 100 mL (control extender). Glutathione were supplemented at the concentrations of 0.5, 1, 2, and 4 mM. The pH (Hanna, model HI-2212, Woonsocket, RI, USA) of the extender was adjusted using sodium bicarbonate buffer to be at 7.2.

Post-thaw evaluation: The frozen straws were thawed in a water bath at 37ºC for 60 seconds, and the contents were expelled into a small warm tube. The general and progressive motility were then evaluated using the ISAS program and assessed for oxidative stress, plasma membrane integrity, morphology defects, acrosome integrity, and viability. Frozen semen evaluation was conducted at the AI Center of the Department of Animal Production and Breeding, Qassim University.

Assessment of sperm motility: The ISAS program (CASA system) was used to assess the motility patterns immediately after diluting the post-thawed semen. A sample (2.7 µL) from each tube was placed onto a warm slide and semen motility was assessed based on five digital images from different fields via a 10× negative-phase contrast objective and warm stage at 38ºC. Motility patterns were measured according to the total motile sperm (TMS %), progressive motility (PM %), curvilinear speed (VCL µm/s), rectilinear speed (VSL µm/s), path velocity (VAP µm/s), linearity index (LIN %), and straightness index (STR %). At least 300 sperm were analyzed from each sample, and the images were read within one second.

Plasma membrane integrity: A hypo-osmotic swelling test (HOST) was used to assess the plasma membrane integrity of the spermatozoa. A minimum of 100 sperm cells were analyzed for the coiled tail using phase-contrast microscopy (400 ×). A mixture of a glucose-based solution at 100 mOsmol and 20 µL of semen was incubated at 37°C for 50 minutes in a water bath (Neild*et al.* 1999).

Viability test: Sperm viability was evaluated using an Acridine Orange (AO) and Propidium Iodide (PI) kit (1:1) (Halotech DNA S.L., Madrid, Spain). First, the semen was diluted to 10-15 × 10^6 sperm/ml. Then 10 µL of diluted semen was placed on a slide. Then 1.0 µL of AO and PI was mixed with the diluted semen. Finally, the mixture was covered and then evaluated using a fluorescence microscope. Living sperm retained the AO, producing green fluorescence, while PI penetrated the damaged sperm, causing red fluorescence. A total of 300 sperm was assessed per sample.

Sperm morphology: Sperm morphology was examined using Hancock's solution. A 15 µL drop of semen was mixed with 15 µL of Hancock's solution in a clean and warm tube. The mixture was dropped and covered, and evaluated under oil immersion at 1000 × magnification. The spermatozoa's morphological defects were classified as sperm abnormalities according to their effect on fertility: major defects include most abnormalities of the head and mid-piece, proximal cytoplasmic droplets, and single abnormalities present in a high percentage, whereas minor defects include looped tails, detached sperm heads, and distal cytoplasmic droplets (Blom, 1983).

Acrosome integrity: A drop of semen sample was mixed with a drop of isotonic Hancock's solution with 6% of formaldehyde on a clean and warmed microscope slide. The mixture was then covered and assessed in a light microscope at 1000 × and at least 200 spermatozoa were assessed for acrosome defects.

Statistical analysis: Descriptive analyses (data were expressed as the means and SE) were determined for the evaluated variables: total motility (TM), progressive motility (PM), path velocity (VAP), curvilinear velocity (VCL), straight line velocity (VSL), straightness (STR), linearity (LIN), wobble (WOB), acrosome integrity, vitality, HOST and morphological defect. Statistical comparisons between groups were performed with one-way analysis of variance (ANOVA) ($P<0.05$). The data were considered statistically different if $p<0.05$ (SPSS, version 21, 2013).

RESULTS

The groups did not demonstrate any significant differences in terms of total motility, progressive motility, and speed metrics (VCL, VSL, and VAP) ($P>0.05$). Nevertheless, the levels of LIN and WOB were signifintly high in the presence of 0.5 mM of glutathione extender, as seen in Table 1. The inclusion of 2 mM glutathione in the extender resulted in a statistically significant increase ($P<0.05$) in the proportion of normally morphology sperm. Furthermore, the acrosome integrity was considerably greater ($P<0.05$) in the extender containing 0.5 mM glutathione compared to the extender containing 1 mM glutathione. Nevertheless, However, the differences were not significant ($P>0.05$) among others. The HOST test showed a statistically significant decrease ($P<0.05$) at the 1 mM glutathione concentration compared to the other values. Furthermore, a concentration of 4 mM of glutathione preserved the integrity of sperm (HOST) at a level comparable to the control. The motility, progressive motility, and VCL (curvilinear velocity) of the semen after thawing were considerably higher ($P<0.05$) in the extender that included 2 mM glutathione, as shown in Table 2 when compared to the other extenders. Nevertheless, there were not any significant differences in VSL, VAP, LIN, and STR across the groups. The incidence of minor abnormalities was significantly higher ($P<0.05$) in the extender containing 4 mM glutathione compared to the other extenders. Conversely, the extender containing 4 mM glutathione exhibited the lowest level of normal sperm morphology compared to the other extenders. The acrosome integrityvalue showed significant differences ($P>0.05$) glutathione (0.5 and 1 mM) and control group compared to others. HOST was highly significant in 0.5 mM glutathione and control group compared to others. However, there was no significant difference in vitality test among group.

DISCUSSION

Glutathione is a tri-peptide consisting of cysteine, glycine, and glutamic acid, with a molecular weight of 307.3g/mol. Glutathione is a crucial coenzyme that plays a vital role in maintaining the equilibrium between prooxidants and antioxidants in the body. It is involved in a broad range of cellular metabolic processes (Bilska*et al.,* 2007).

Table 1. Effect of adding glutathione at different concentrations on stallion's sperm motility parameters, acrosome integrity, HOST, and morphology in cooled semen (Mean ± SE)

Item	Control	Glutathione (mM)			
		0.5	1	2	4
Total motility (%)	86.25 ± 1.56	80.00 ± 2.88	85.71 ± 2.02	83.33 ± 3.33	84.54 ± 1.57
Progressive motility (PM) (%)	31.66 ± 8.96	28.00 ± 3.35	26.53 ± 3.61	36.42 ± 3.04	35.35 ± 6.54
VCL (µm/s)	89.33 ± 2.13	88.88 ± 3.27	90.23 ± 4.09	84.51 ± 3.40	87.22 ± 4.01
VSL (µm/s)	22.43 ± 0.49	27.48 ± 2.89	24.03 ± 1.94	23.14 ± 1.47	22.51 ± 3.54
VAP (µm/s)	46.58 ± 1.38	48.01 ± 2.68	46.20 ± 1.50	44.08 ± 1.70	44.10 ± 1.76
LIN (%)	26.34 ± 0.52^{b}	31.65 ± 2.87^{a}	27.23 ± 1.28^{ab}	27.55 ± 0.99^{ab}	26.42 ± 1.54^{ab}
STR (%)	52.48 ± 0.43	57.53 ± 4.66	52.11 ± 2.81	51.41 ± 1.94	51.18 ± 2.63
WOB (%)	52.33 ±0.33^{ab}	54.81 ± 1.19^{a}	52.26 ± 0.43^{ab}	52.73 ± 0.29^{ab}	51.50 ± 1.05^{b}
Minor abnormalities (%)	2.95 ± 1.75^{ab}	3.88 ± 0.97^{ab}	4.62 ± 0.88^{a}	3.01 ± 0.42^{ab}	2.44 ± 0.21^{b}
Major abnormalities (%)	16.17 ± 1.79	13.53 ± 1.23	16.55 ± 0.74	14.10 ± 1.32	14.87 ± 1.45
Normal morphology (%)	82.35 ±2.32^{ab}	82.93 ± 1.62^{ab}	78.81 ± 0.58^{b}	83.70 ± 1.42^{a}	82.81 ± 1.47^{ab}
Acrosome integrity (%)	86.50 ±1.04^{ab}	88.04 ± 1.13^{a}	84.21 ± 0.78^{b}	85.76 ± 0.95^{ab}	85.94 ± 1.11^{ab}
HOST (%)	72.21 ± 5.24^{a}	66.33 ± 3.16^{a}	51.09 ± 0.66^{b}	63.08 ± 5.00^{a}	73.13 ± 2.08^{a}

^{a,b} Values with different superscripts in the same row differ significantly at $P<0.05$.

Table2. Effect of adding glutathione at different levels on stallion's sperm motility parameters, acrosome integrity, HOST, and morphology in post-thaw frozen semen (Mean ± SE)

Item	Control	Glutathione (mM)			
		0.5	1	2	4
Total motility (%)	38.00 ± 1.22^{b}	40.00 ± 3.08^{b}	45.00 ± 2.23^{b}	61.25 ± 3.14^{a}	45.00 ± 1.82^{b}
Progressive motility (PM) (%)	18.33 ± 1.85^{b}	16.90 ± 2.56^{b}	15.66 ± 1.27^{b}	28.13 ± 2.07^{a}	16.18 ± 1.65^{b}
VCL (µm/s)	87.45 ±1.30^{ab}	82.05± 0.58^{b}	87.53 ± 0.76^{ab}	90.03 ± 1.18^{a}	81.52 ± 3.14^{b}
VSL (µm/s)	22.71 ± 0.19	25.11 ± 2.81	24.76 ± 1.72	26.97 ± 3.09	24.96 ± 3.08
VAP (µm/s)	45.23 ± 0.49	43.23 ± 1.64	43.86 ± 0.79	47.11 ± 1.40	43.79 ± 1.85
LIN (%)	26.65 ± 0.20	31.11 ±2.32	28.88 ± 1.63	30.91 ± 3.70	31.79 ± 3.55
STR (%)	50.63 ± 0.08	57.45 ± 4.14	56.65 ± 3.59	57.01 ± 4.57	56.87 ± 4.56
WOB (%)	52.33 ± 0.54^{ab}	53.90 ±0.20^{ab}	50.98 ± 0.50^{b}	53.35 ± 2.27^{ab}	55.20 ± 1.50^{a}
Minor abnormalities (%)	9.87 ± 0.79^{b}	11.35 ±1.32^{ab}	9.85 ± 2.12^{b}	11.23 ± 0.75^{ab}	15.38± 2.06^{a}
Major abnormalities (%)	8.53 ± 0.29	8.79 ± 0.50	11.92 ± 2.75	8.65 ±1.23	9.62 ± 1.39
Normal morphology (%)	81.81 ± 1.00^{a}	79.84 ± 1.34^{a}	78.22 ± 0.97^{ab}	80.07 ± 1.30^{a}	74.99 ± 1.01^{b}
Acrosome integrity (%)	93.87 ± 0.60^{a}	94.53 ± 0.56^{a}	93.22 ± 2.66^{a}	84.27 ± 0.69^{b}	88.1 ± 1.72^{b}
HOST (%)	51.48 ± 0.95^{a}	54.04 ± 1.28^{a}	39.72 ± 1.85^{b}	43.29 ± 8.27^{b}	46.32 ± 5.30^{ab}
Vital test (%)	42.71 ± 11.54	33.96 ± 2.40	40.20± 6.68	36.41 ± 5.90	37.22 ± 5.46

^{a,b} Values with different superscripts in the same row differ significantly at $P<0.05$.

The primary processes that govern the function of glutathione at the cellular level are primarily its thiol (-SH) group, which attaches to proteins that are removed under oxidative stress, and its involvement in safeguarding cell membranes against lipid peroxidation (Lenzi*et al.,* 1994). Glutathione is present in both the sperm and seminal plasma and acts as a protective barrier against the build-up of harmful free radicals that occur during the normal process of sperm movement during mating. In the present investigation, glutathione has shown a positive effect on enhancing both total motility and progressive motility. De Oliveira *et al.,* (2013) reported that the addition of glutathione at a concentration of 2.5 mM to the freezing extender used in the cryopreservation of fertile and subfertile stallions' sperm resulted in an improvement in total motility. However, there were no significant differences observed in the other parameters studied, including progressive motility, membrane integrity, acrosome, and DNA. Nevertheless, Baumberand colleagues (2005) did not see any enhancement in both overall and gradual movement with the inclusion of 10 mM glutathione. The same antioxidants added to refrigerated horse semen had comparable outcomes, as reported by Ball *et al.,* (2001). Regarding the preservation of semen, the inclusion of glutathione at a concentration of 2 mmol in the diluent for dairy goat semen enhances the quality of sperm after thawing, as shown by Zou*et al.*, (2021). The present investigation yielded data that included TM, PM, and normal morphology. Glutathione was used in the HF-20 extender at a concentration of 2 mM/100 ml. In addition, Ogata *et al.,* (2022) experimented to examine the impact of including four different concentrations of reduced glutathione (0, 1, 5, and 10 mM) in a bull semen extender. They observed that the inclusion of 5 mM glutathione not only boosted the post-thaw sperm properties but also improved its capacity to fertilize oocytes in a laboratory setting.

In addition, the addition of 0.5mM of glutathione to the extender for cold storage (5°C) for 72 hours resulted in improved sperm characteristics in cooled bull semen (Munsi*et al.,* 2007). According to research conducted by Zeitoun and Al-Damegh(2015), augmenting ram semen extender with glutathione at a concentration of 1-2 mM per ml resulted in increased survivability and progressive motility of cooled sperm. Nevertheless, the correlation between spermatic movement and fertility is not always reliable, since sperm motility is only one of the many essential requirements for a spermatozoon to successfully fulfill its biological role, which is the fertilization ofoocytes (Graham and Moce, 2005). There were no discernible differences in acrosome integrity, HOST, and vitality between the test group and the control group. The findings of this investigation, similar to the research conducted by Yánez-Ortiz *et al.* (2021), revealed that the addition of glutathione at concentrations ranging from 0 to 10 mM to a donkey semen extender formulated for freezing did not enhance sperm quality compared to the control group. In this research, a notable decrease in the integrity of the sperm membrane (plasmatic and acrosome) was observed, which is a predictable outcome after the freezing process. The process of freezing, as explained by Mazur (1984), results in the creation of ice crystals, which causes an elevation in the concentration of salt in the unfrozen portion. Elevated levels of salt may lead to the dehydration of sperm cells, resulting in their deformation and causing harm to the structure of their membranes as well as the denaturation of proteins. As a result, when spermatozoa are frozen, they experience permanent harm that is marked by aberrant movement (circular or retrograde), a quick decrease in motility, damage to the acrosome and plasmatic membrane, reduced metabolism, and loss of intracellular components (Amann and Pickett, 1987). Nevertheless, the inclusion of 2 mM

glutathione was deemed to be significantly effective in maintaining sperm velocity when compared to the other treatments.

CONCLUSION

Supplementation of semen extender with glutathione has a beneficial effect on sperm motility, speed parameters, and antioxidant activity of Arabian stallion spermatozoa, particularly after cryopreservation in a dose-dependent manner. These positive effects seemed to be due to anit's role in protecting cell membranes from lipid peroxidation of the cryopreserved spermatozoa. The addition of glutathione at 0.5-2mM/100 mL to the stallion semen extender seems to be the best concentration resulting in the best post-thaw sperm characteristics for further fertility trials.

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