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# Effect of Glutathione (GSH) on Microscopic Parameters and DNA Integrity in Egyptian Buffalo Semen During Liquid and Frozen Storage

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**Abstract:** The current study was performed to test the of quality as well as the DNA integrity of preserved liquid and frozen buffalo spermatozoa due to addition of different concentrations of glutathione (0.50, 1.00, 2.00 and 3.00 mM). Liquid semen was preserved for 120 hour with or without addition of glutathione and examined for progressive motility, viability, plasma and acrosomal membranes integrity at 24, 48, 72, 96 and 120 hr of storage. Frozen-thawed semen was examined for the same criteria after thawing and during incubation for 9 hours. DNA integrity was assessed in fresh, cooled and frozen-thawed semen with or without addition of glutathione. Individual motility, viability, intact acrosomal and plasma membranes of stored spermatozoa were significantly ($P<0.01$) improved by inclusion of glutathione in the semen extender. The post-thaw sperm motility, viability, plasma and acrosomal membranes integrity were significantly higher ($P<0.01$) in samples treated with 0.50 mM and 1.00 mM glutathione than those of untreated spermatozoa. Addition of exogenous glutathione to the semen extender significantly ($P<0.01$) decreases the damaged DNA in frozen-thawed semen especially at concentration of 0.50 or 1.00 mM compared to control and 2.00 or 3.00 mM glutathione. The higher pregnancy rates ((60% and 55%) were obtained for semen samples treated with 0.50 and 1.00 mM, respectively. These results indicate that, addition of 0.50 or 1.00 mM GSH to semen diluent improve the keeping quality of liquid semen up to 120 hr, significantly improve sperm characteristics, reduce DNA damage following freezing and thawing and improve the fertility of frozen-thawed buffalo semen.

**Key words:** Glutathione % Semen % Frozen % Buffalo % DNA

## INTRODUCTION

Water buffaloes (Bubalus bubalis) represent a major livestock in tropics where, apart from being used as draft animals, they produce primarily milk or meat for human consumption [1]. Both types (swamp and river) of buffaloes are used for natural mating. Today, there is increasing trend to use artificial insemination (AI) as a breeding tool, for which semen is usually collected from selected sires and processed for production of frozen AI doses. This development has increased exponentially in river type buffalo which is present in Egypt [2]. Buffaloes constitute a significant part of the domestic stock in Egypt. The increasing importance of buffaloes in the dairy industry of Egypt has made artificial insemination technology a requisite to improve productivity of this animal species. Successful artificial insemination required high quality cryopreserved semen.

Application of AI with frozen-thawed semen has been reported at limited scale in buffalo, because of poor freezability and fertility of buffalo bull spermatozoa as compared to cattle bull spermatozoa [3] as buffalo spermatozoa are more susceptible to hazards during freezing than cattle spermatozoa [4]. These hazards could be minimized by optimizing the cooling and freezing rates and using appropriate diluting media in which spermatozoa are frozen [5]. The fertilizing capacity of spermatozoa in preserved semen (chilled or frozen) is important for successful artificial insemination programme [6]. The cooling and freeze thawing processes produce physical and chemical stresses on the sperm membrane that reduce sperm viability and fertilizing ability [7]. The fertilizing capacity of chilled semen, decrease with storage time [8]. Moreover, the morphology and motility of spermatozoa are too low after 2 days of chilling [9]. Cooling and freezing of semen are associated with

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oxidative stress and reactive (ROS) oxygen species [10]. Among ROS-induced damages to sperm there are those mediated by oxidative reaction of spermatozoal phospholipids-bound polyunsaturated fatty acids, leading to lipid peroxidation [11]. During storage of semen, sperm phospholipids undergo peroxidation, leading to the formation of toxic fatty acids and hence structural damage to the sperm cell accompanied by decreased motility and metabolism [12]. In addition to the reduction of sperm-egg fusion [13] and DNA damage [14]. Semen has a delicate balance between the antioxidant potential of seminal plasma and sperm cell and the pro oxidant activities of spermatozoal metabolism, particularly active in non-physiological conditions such as during *in vitro* manipulation, determines the overall rate of spermatozoal lipid peroxidation [7]. Glutathione (GSH) is one of the non-enzymatic antioxidants, tripeptide distributed in living cells which play an important role in the intracellular protective mechanism against oxidative stress as it can react with many ROS and act as cofactor for glutathione peroxidase that catalyses the reduction of toxic $H_2O_2$ and hydroperoxides [15]. The resulting oxidized glutathione (GSSG) is reduced to GSH by glutathione reductase using NADPH as the co-factor. The GSH/GSSG pair plays important roles as protective agents against ROS-induced damages in many cell types [16]. Addition of glutathione to frozen bull semen improves sperm motility [17]. Besides the routine sperm parameters, the evaluation of spermatozoa DNA integrity could add important data on the quality of spermatozoa and reproductive potential [18]. Therefore, the objectives of the current study were to assess the effect of exogenous GSH on sperm motility, viability, plasma membrane integrity, acrosomal membrane integrity and DNA integrity of chilled and frozen buffalo semen as well as fertility of frozen-thawed semen.

## MATERIALS AND METHODS

All materials utilized were purchased from Sigma-Aldrich. Company (USA) unless stated otherwise.

**Semen Collection and Preservation:** Semen samples were collected twice a week by an artificial vaginal from five fertile, normal buffalo bulls (4-6 years) maintained at Mehalat Moussa Research Farm belonging to Animal Production Research Institute, Egypt. Immediately after collection, semen samples were transferred to the laboratory and kept in a water bath at 30°C for evaluation. Semen samples with more than 70% progressive motility and 600 x $10^6$/ml sperm cell concentration were pooled and extended in a tris-egg yolk glycerol extender [Tris (hydroxymethyl amino methane), 3.028 g; citric acid monohydrate 1.675 g; fructose 1.25 g; penicillin G sodium 1000 IU/ml; streptomycin sulphate, 1000 µg/ml; double distilled water up to 100 ml] with 20% egg yolk and 7% glycerol to yield approximately 60 x $10^6$ motile sperm cells/ml. The pH of the buffer was maintained from 6-8 to 7.00 and the final extender pH was 7.00. The pooled semen was divided into five aliquots of equal volumes and diluted with a tris egg yolk extender provided with 0 (control), 0.50, 1.00, 2.00 and 3.00 mM glutathione at 30°C [6]**.** The extended semen was cooled and equilibrated in an equilibration chamber for 4 h at 5°C before filling in 0.5 ml straws. After filling and sealing the straws were placed in a rack of 5 cm above liquid nitrogen in the vapour phase for 10 min and plunged into liquid nitrogen tank (-196°C). For liquid preservation, semen was diluted to 10 million sperm/ml in tris egg yolk glycerol extender and cooled to 5°C over a period of 2 h., preserved at 4°C for 120 h.

**Semen Evaluation**

**Chilled Semen:** Progressive sperm motility was assessed subjectively under a phase contrast microscope (400 x) equipped with a heated stage adjusted to 37°C. The motility was examined after dilution as well as after 24, 48, 72, 96 and 120 h of the storage period.

Sperm viability (live-dead) was determined using eosin-nigrosin stain [19]**.** The smears were prepared by mixing one drop of semen sample with two drops of the stain on a warm slide and immediately spreading the stain with one edge of a second slide. The viability was assessed by counting 200 spermatozoa under a phase contrast microscope (400 x).

**Acrosomal Membrane Integrity:** A 250 µl of each semen sample was fixed in 25 µl of 1% formal citrate (99ml sodium citrate dehydrate 2.9% and 1 ml commercial formaldehyde 37%). Two hundred spermatozoa were counted under a phase-contrast microscope (100 x) for their normal apical ridge. Abnormalities likes absent, ruffled and swollen acrosome were counted [20].

**Plasma Membrane Integrity:** The hypo-osmotic swelling (HOSt) test was used to evaluate the functional integrity of the sperm membrane based on the curled and swollen tails. This was performed by incubating 10 µl semen in 100 µl of 100 m Osm/k hypo-osmotic solution (0.735 g sodium citrate and 1.351 g fructose at 37°C for 45 min. After incubation the sample was gently mixed and 0.1 ml of the mixture was spread with a cover slip on a warm slide. A total of 200 sperm cells were counted (x400) under a phase-contrast microscope. Spermatozoa with swollen or coiled tails were recorded [21].

The evaluation of motility, viability, acrosomal and plasma membranes integrity were repeated daily after cooling up to five days.

**Frozen Thawed Semen:** Frozen thawed semen was evaluated for progressive sperm motility, sperm viability, acrosomal as well as plasma membrane integrity during prefreeze and post-thawing. Frozen thawed semen was kept in a $CO_2$ incubator at 37°C without further dilution and examined after 3, 6 and 9 h, with the time of thawing considered to be 0 hour [22].

**Sperm DNA Integrity:** Acridine-orange (AO) staining assay was used for determination of sperm DNA integrity [23]. Air-dried slides were fixed overnight in freshly prepared Carnoy's solution (three parts methanol and one part glacial acetic acid) and allowed to air dry for a few min. Dried slides were stained for 3 min. with AO. The stained slides were evaluated immediately under fluorescence microscope with 490 nm excitation light and 530 nm barrier filter. Normal DNA showed green fluorescence over the head region, while DNA abnormalities showed varying fluorescence (from yellow-green to red). At least 100 spermatozoa per smear were evaluated for DNA abnormalities. DNA integrity was evaluated in fresh, cooled and frozen thawed semen.

**Fertility Test:** A fertility test is undertaken to explore if the improvement in post-thaw sperm motility observed due to addition of glutathione would be resulted in an improvement in the pregnancy rates. Therefore, frozen-thawed semen from the control and treated (0.50, 1.00 mM) samples were used for artificial insemination. A total number of 60 Egyptian buffalo-cows (4-8 years, 560 kg body weight) were used in the fertility test (allocated in three groups each of 20 animals). Estrous was synchronized using a double intramuscular administration of 500 µg (2ml) per dose cloprostenol (Estrumate, Coopers Animal Health Limited, Pharma Egypt Trading) at 11- day interval. Estrous was detected twice a day by a well trained herd man. However, special attention was paid for acceptance of buffalo-cows to buffalo-bulls as standing of buffalo cows to be mounted by the bull is the most reliable sign of estrous in buffaloes [24]. The day at which the female stand to be mounted was considered the day of estrous. At the time (12 hours of estrous detection) of insemination, one semen straw used for insemination. After 40 days of insemination, the buffalo-cows were examined for pregnancy by palpation per rectum. Pregnancy rate was defined as the number of pregnant buffalo-cows over the number of inseminated buffalo-cows.

**Statistical Analysis:** The obtained data were analyzed using SAS computed system. Analysis of variance was done for the percentage data after transforming the data by arcsine transformation as described previously [25]. The treatment mean values were compared by least significant difference (LSD) adjusted by Duncan's multiple range test [26]. The data were expressed as means + SEM. The pregnancy rates were compared using chi-square test.

## RESULTS

Table 1 and 2 show the impact of glutathione on the individual motility and viability of chilled buffalo semen in tris egg yolk diluent. It was clear that individual motility and viability of stored spermatozoa were significantly ($P<0.01$) improved by inclusion of glutathione in the semen extender. The highest values for sperm motility at 120 h of storage (58.30±1.13 and 57.30± 1.11%) and viability (67.50±0.42 and 66.90± 0.40%) were achieved after exposure of spermatozoa to 0.50 and 1.00 mM glutathione compared to 0.00, 2.00 and 3.00 mM. The mean values of intact plasma and acrosomal membranes in treated and control semen samples are depicted in Table 3 and 4. The mean values of intact plasma and acrosomal membranes were significantly higher ($P<0.01$) at 120 hr of incubation at 5°C in 0.50 and 1.00 mM GSH treated samples than other treated and control samples.

Table 1: Sperm motility (%) of Egyptian buffalo semen supplemented with different concentrations of glutathione (GSH) for different storage times at 5°C (Means±SEM ;n=20)

| | Storage time (h) | | | | | |
|---|---|---|---|---|---|---|
| Glutathione conc. (mM) | 0 | 24 | 48 | 72 | 96 | 120 |
| 0.50 | 79.80±0.73 | 74.30$^{a}$±0.81 | 72.30$^{a}$±0.83 | 66.30$^{a}$±0.70 | 63.30$^{a}$±0.74 | 58.30$^{a}$±1.13 |
| 1.00 | 78.00±0.64 | 75.30$^{a}$±0.90 | 72.50$^{a}$±0.75 | 65.30$^{a}$±0.73 | 62.50$^{a}$±0.75 | 57.30$^{a}$±1.12 |
| 2.00 | 78.00±0.65 | 73.80$^{b}$±0.78 | 65.50$^{b}$±0.86 | 54.00$^{b}$±0.76 | 47.30$^{b}$±0.62 | 42.30$^{b}$±0.94 |
| 3.00 | 77.80±0.62 | 72.20$^{a}$±0.85 | 64.50$^{b}$±0.87 | 52.80$^{b}$±0.75 | 47.00$^{b}$±0.70 | 41.80$^{b}$±0.88 |
| Control | 79.30±0.73 | 75.80$^{a}$±0.79 | 66.80$^{b}$±0.81 | 55.30$^{b}$±0.75 | 45.30$^{b}$±0.73 | 38.00$^{b}$±0.68 |

a, b: different superscripts within the same column are significantly different ($P<0.01$).

Table 2: Sperm viability (%) of Egyptian buffalo semen supplemented with different concentrations of glutathione (GSH) for different storage times at 5°C. (Means±SEM; n=20)

| | Storage time (h) | | | | | |
|---|---|---|---|---|---|---|
| Glutathione conc. (mM) | 0 | 24 | 48 | 72 | 96 | 120 |
| 0.50 | 89.70±0.70 | 84.70$^{a}$±0.49 | 83.50$^{a}$±0.46 | 76.80$^{a}$±0.43 | 73.80$^{a}$ ±0.37 | 67.50$^{a}$±0.42 |
| 1.00 | 89.00±0.70 | 84.40±0.60 | 84.70$^{a}$±0.88 | 77.30$^{a}$±0.65 | 73.20$^{a}$±0.58 | 66.40$^{a}$±0.40 |
| 2.00 | 89.20±0.75 | 84.50±0.53 | 78.10$^{b}$±0.40 | 72.80$^{b}$±0.46 | 63.40$^{b}$±0.50 | 54.80$^{b}$±0.53 |
| 3.00 | 89.80±0.49 | 84.40±0.54 | 79.20$^{b}$±0.41 | 73.20$^{b}$±0.56 | 63.10$^{b}$±0.41 | 53.60$^{b}$±0.37 |
| Control | 88.60±0.75 | 83.70±0.46 | 76.60$^{b}$±0.36 | 72.10$^{b}$±0.40 | 62.80$^{b}$±0.35 | 55.10$^{b}$±0.39 |

a, b: different superscripts within the same column are significantly different (P<0.01).

Table 3: Intact plasma membrane (%) of Egyptian buffalo semen supplemented with different concentrations of glutathione (GSH) for different storage times at 5°C. (Means±SEM; n=20)

| | Storage time (hr) | | | | | |
|---|---|---|---|---|---|---|
| Glutathione conc. (mM) | 0 | 24 | 48 | 72 | 96 | 120 |
| 0.50 | 54.50±0.38 | 50.90±0.43 | 48.20$^{a}$±0.50 | 46.90$^{a}$±0.37 | 42.30$^{a}$±0.52 | 39.80$^{a}$±0.36 |
| 1.00 | 53.10±0.40 | 51.00±0.47 | 47.80$^{a}$±0.44 | 46.80$^{a}$±0.40 | 42.40$^{a}$±0.50 | 39.60$^{a}$±0.39 |
| 2.00 | 52.60±0.38 | 50.70±0.42 | 41.00$^{b}$±0.49 | 40.80$^{b}$±0.37 | 33.10$^{b}$±0.50 | 23.20$^{b}$±0.42 |
| 3.00 | 53.60±0.44 | 51.30±0.37 | 42.20$^{b}$±0.67 | 41.10$^{b}$±0.30 | 33.00$^{b}$±0.51 | 22.00$^{b}$±0.32 |
| Control | 54.00$^{a}$±0.40 | 49.10$^{a}$±0.57 | 39.40$^{b}$±0.41 | 39.60$^{b}$±0.43 | 30.60$^{b}$±0.56 | 22.50$^{b}$±0.37 |

a, b: different superscripts within the same column are significantly different (P<0.01).

Table 4: Intact acrosome (%) of Egyptian buffalo semen supplemented with different concentrations of glutathione (GSH) for different storage times at 5°C (Means±SEM; n=20)

| | Storage time (hr) | | | | | |
|---|---|---|---|---|---|---|
| Glutathione conc. (mM) | 0 | 24 | 48 | 72 | 96 | 120 |
| 0.50 | 92.50±0.42 | 87.30±0.41 | 85.60$^{a}$±0.41 | 86.00$^{a}$±0.40 | 82.90$^{a}$±0.62 | 79.50$^{a}$±0.53 |
| 1.00 | 92.80±0.41 | 87.20±0.42 | 85.10$^{a}$±0.44 | 85.90$^{a}$±0.40 | 83.30$^{a}$±0.59 | 78.50$^{a}$±0.55 |
| 2.00 | 92.60±0.37 | 86.60±0.42 | 82.20$^{b}$±0.53 | 81.80$^{b}$±0.37 | 72.20$^{b}$±0.52 | 64.40$^{b}$±0.52 |
| 3.00 | 92.30±0.39 | 86.10±0.38 | 81.80$^{b}$±0.47 | 81.30$^{b}$±0.42 | 72.60$^{b}$±0.55 | 63.10$^{b}$±0.49 |
| Control | 91.70±0.45 | 86.80±0.44 | 81.40$^{b}$±0.46 | 78.00$^{b}$±0.36 | 70.40$^{b}$±0.54 | 62.60$^{b}$±0.46 |

a, b: different superscripts within the same column are significantly different (P<0.01).

Table 5: Motility and viability percentages of frozen thawed buffalo spermatozoa preserved in tris egg yolk extender supplemented with 0.5 or 1.00 mM glutathione.(Mean±SEM ;n=15)

| Parameter | Individual motility (%) | | | Viability (%) | | |
|---|---|---|---|---|---|---|
| Glutathione conc. mM | After dilution | Before freezing | After thawing | After dilution | Before freezing | After thawing |
| 0.5 | 80.00±0.64 | 79.00$^{a}$±0.67 | 47.33$^{a}$±0.77 | 85.10±0.52 | 83.30$^{a}$±0.75 | 56.70$^{a}$±0.49 |
| 1.00 | 80.00±0.64 | 80.00$^{a}$±1.02 | 46.67$^{a}$±0.88 | 85.30±0.46 | 82.60$^{a}$±0.58 | 55.70$^{a}$±0.47 |
| Control | 81.00±0.67 | 74.67$^{b}$±0.69 | 39.00$^{b}$±0.93 | 84.90±0.31 | 75.60$^{b}$±0.53 | 45.10$^{b}$±0.57 |

a, b: different superscripts within the same column are significantly different (P<0.01).

Table 6: Effect of exogenous glutathione on the plasma and acrosomal membranes integrity (%) of frozen thawed buffalo spermatozoa (Means + SEM; n=15)

| Parameter | Intact plasma membrane (%) | | | Intact acrosome (%) | | |
|---|---|---|---|---|---|---|
| Glutathione conc. mM | After dilution | Before freezing | After thawing | After dilution | Before freezing | After thawing |
| 0.5 | 73.80±0.49 | 68.10$^{a}$±0.71 | 63.50$^{a}$±0.67 | 92.80±0.45 | 86.30$^{a}$±0.58 | 63.30$^{a}$±0.46 |
| 1.00 | 73.10±0.45 | 69.50$^{a}$±0.58 | 64.10$^{a}$±0.52 | 92.30±0.42 | 86.90$^{a}$±0.45 | 64.30$^{a}$±0.49 |
| Control | 72.10±0.53 | 63.70$^{b}$±0.70 | 51.30$^{b}$±0.56 | 91.50±0.37 | 70.70$^{b}$±0.64 | 44.40$^{b}$±0.39 |

a, b: different superscripts within the same column are significantly different (P<0.01).

Table 7: Effect of glutathione (mM) on the motility and viability of frozen thawed buffalo spermatozoa (Mean + SEM) incubated *in vitro* for a period of 9 h

| | Motility (%) | | | Viability (%) | | |
|---|---|---|---|---|---|---|
| Duration (hr) | Control | 0.5 mM | 1.0 Mm | Control | 0.5 mM | 1.0 mM |
| 0 | $39.00^{b}\pm0.93$ | $47.30^{a}\pm0.77$ | $46.70^{a}\pm0.87$ | $45.10^{b}\pm0.57$ | $56.30^{a}\pm0.49$ | $55.70^{a}\pm0.47$ |
| 3 | $35.70^{a}\pm0.89$ | $35.30^{a}\pm0.71$ | $36.30^{a}\pm0.85$ | $41.80^{b}\pm0.42$ | $54.50^{a}\pm0.68$ | $53.50^{a}\pm0.57$ |
| 6 | $9.00^{b}\pm0.67$ | $10.70^{a}\pm0.62$ | $13.30^{a}\pm0.59$ | $20.30^{b}\pm0.45$ | $26.50^{a}\pm0.59$ | $25.90^{a}\pm0.50$ |
| 9 | $0.0^{b}\pm0.0$ | $2.67^{a}\pm0.62$ | $4.67^{a}\pm0.81$ | $6.47^{b}\pm0.48$ | $15.60^{a}\pm0.54$ | $15.10^{a}\pm0.69$ |

a, b: different superscripts within the same row are significantly different (P<0.01). (n=15)

Table 8: Effect of glutathione (mM) on plasma and acrosomal membranes integrity of frozen thawed buffalo spermatozoa (Mean + SEM; n=15) incubated *in vitro* for a period of 9 h

| | Intact acrosome (%) | | | | | |
|---|---|---|---|---|---|---|
| Intact plasma membrane (%) | | | | | | |
| Duration (hr) | Control | 0.5 mM | 1.0 mM | Control | 0.5 mM | 1.0 mM |
| 0 | $51.30^{b}\pm0.56$ | $65.10^{a}\pm0.61$ | $63.40^{a}\pm0.60$ | $44.40^{b}\pm0.39$ | $63.30^{a}\pm0.46$ | $64.30^{a}\pm0.49$ |
| 3 | $45.90^{b}\pm0.54$ | $53.30^{a}\pm0.45$ | $56.80^{a}\pm0.69$ | $34.10^{b}\pm0.53$ | $51.90^{a}\pm0.59$ | $55.50^{a}\pm0.43$ |
| 6 | $36.00^{b}\pm0.62$ | $42.70^{a}\pm0.79$ | $44.80^{a}\pm0.72$ | $20.40^{b}\pm0.64$ | $41.60^{a}\pm0.50$ | $44.50^{a}\pm0.60$ |
| 9 | $15.30^{b}\pm0.55$ | $19.50^{a}\pm0.54$ | $21.40^{a}\pm0.49$ | $10.10^{b}\pm0.37$ | $20.50^{a}\pm0.35$ | $23.70^{a}\pm0.47$ |

a, b: different superscripts within the same row are significantly different (P<0.01)

As presented in Table 5, after freeze/thaw processing of buffalo semen in tris egg yolk glycerol diluent, the mean percentage of post-thaw motility and viability of spermatozoa treated with 0.50 mM and 1.00 mM glutathione were significantly (P<0.01) higher than that of untreated spermatozoa. Moreover, statistical analysis did not reveal any significant variation among post thaw sperm motility and viability percentages of semen presented in tris egg yolk diluent containing 0.50 or 1.00 mM glutathione. The sperm motility and viability did not vary significantly between post-dilution and pre-freeze semen samples. The effect of glutathione on the percentage of intact plasma and acrosomal membranes is presented in Table 6. There were non-significant differences in the percentages of intact plasma or acrosomal membranes between the post-dilution and prefreeze semen samples in treated and control samples. Furthermore, there was highly (P<0.01) significant variation between post-thawing and post-dilution semen samples. The percentages of post-thawing plasma and acrosomal membranes integrity were significantly higher (P<0.01) in glutathione treated (0.50 and 1.00 mM) than control semen samples. However, there were non-significant differences in the percentages of intact plasma and acrosomal membranes between 0.50 and 1.00 mM treated samples (Table 6).

The post-thawing (0h) sperm motility, viability, intact plasma and acrosomal membranes (Table 7, 8) were significantly (P<0.01) higher in 0.50 and 1.00 mM glutathione treated groups than the control group. There was significant (P<0.01) decrease in all studied parameters due to the time of storage with significant higher values for 0.50 and 1.00 mM glutathione compared to the control.

Figure 1 explicates the effect of glutathione as well as freezing-thawing processes on the DNA integrity of buffalo spermatozoa. Post-thaw semen demonstrated the highest rate (6.80±0.56%) of damaged DNA than fresh (1.50±0.40), diluted (1.70±0.50) and cooled (2.30±0.62) samples. Addition of exogenous glutathione to the tris egg yolk extender significantly (P<0.01) decreases the percentage of damaged DNA in frozen thawed semen especially at concentration of 0.50 or 1.00 mM compared to control and 2.00 or 3.00 mM glutathione (2.50±0.40, 2.40± 0.38 vs. 6.80±0.56, 3.20± 0.52 and 3.40±0.48) for 0.50 mM, 1.00 mM control, 2.00 mM and 3.00 mM, respectively (Figure 1).

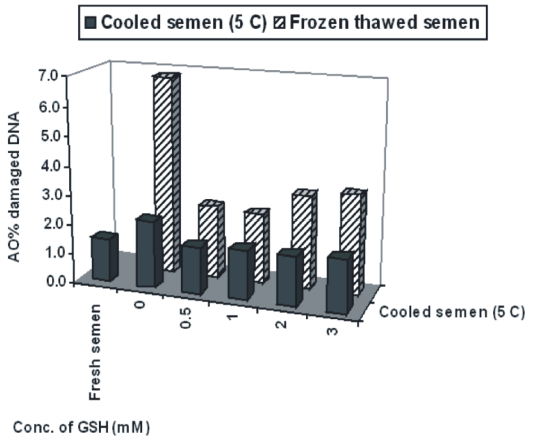


Fig. 1: Effect of exogenous glutathione on the DNA integrity of buffalo spermatozoa

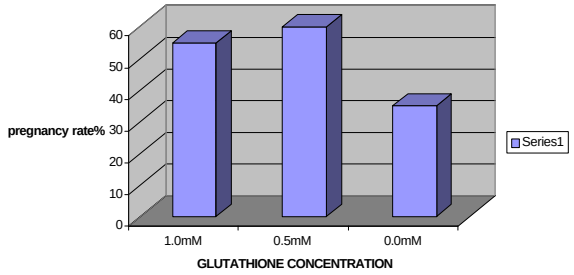


Fig. 2: Effect of glutathione on fertility of frozen- thawed buffalo semen

**Fertility Test:** The outcomes of the artificial insemination with frozen-thawed semen are delineated in Figure 2. The over all pregnancy rate was 50.00%. The pregnancy rates obtained with glutathione treated semen 0.50 mM (60%) or 1.00 mM (55%) were significantly higher ($\times^2$= 8.99) than that obtained with egg yolk glycerol extender without GSH (35%). Statistical analysis did not reveal any significant variation among the pregnancy rates of GSH (0.50, 1.00 mM) treated samples. ($\times^2$= 1.34).

## DISCUSSION

The high concentration of polyunsaturated fatty acids in the spermatozoa, render the sperm susceptible to lipid peroxidation due to oxidative stress induced by freezing and thawing. In addition, the antioxidant system of seminal plasma and spermatozoa is compromised during semen processing [11]. Therefore, inclusion of exogenous antioxidants may modulate the antioxidant system of semen. The results of the present study showed that addition of 0.50 mM or 1.00 mM GSH improve the keeping quality (up to 5 days) of buffalo semen presented at 5°C. The sperm motility was declined by the time of storage and remained over 50% for up to 5 days after addition of 0.50 or 1.00 mM glutathione. In contrast, the decline rate in the motility percentage was higher in semen samples treated with 2.00 or 3.00 mM GSH or without GSH. It has been reported that the quality of chilled semen decreased with time and remained suitable for use up to 2 days as judged by motility and morphology [9, 27].The improvement of semen quality due to addition of exogenous glutathione recorded in the present study was previously reported in bull semen in the form of motility and intact acrosomal membrane [6]. Moreover, the addition of exogenous GSH was significantly improve the percentages of sperm viability and intact plasma membrane (swelling tails) especially at a level of 0.50 or 1.00 mM GSH. The highest percentages of intact plasma and acrosomal membranes which were found in the present experiment due to 0.50 or 1.00 mM glutathione may be the reason for better motility in these samples [28].

GSH helps maintaining the integrity of normal acrosome [29] and stabilize the plasmalemma of spermatozoa and so increase motility. Oxidative stress is associated with lipid peroxidation of the sperm outer membrane which leads to loss of sperm motility [30] decreased fertilization capacity [31] and increased chromatin damage [32]. In the present investigation, supplementation of the cryopreservation medium with 0.50 or 1.00 mM glutathione resulted to significant increase in the sperm quality characteristics like post-thaw motility, viability, membrane and acrosome integrity. Previous study [33] suggested that lipid peroxidation and subsequent membrane damage is at its peak during thawing. Glutathione protect the spermatozoa from this damage by inhibiting the lipid peroxidation process. The spermatozoa are readily undergoing lipid peroxidation, particularly in the presence of oxygen. Addition of antioxidant like GSH might have beneficial effects on sperm [17]. The significant improvement on the post-thaw sperm motility and viability recorded herein due to the addition of 0.50 or 1.00 mM glutathione is similar to that recorded previously [34] due to the addition of glutamine which is an effective precursor for GSH synthesis. The supplementation of the extender with 0.50 or 1.00 mM GSH resulted in higher intact plasma and acrosomal membranes compared to the control samples. These findings were similar to those previously reported for the frozen-thawed sperm of the stallion [35]. GSH may therefore provide cryoprotection in mammalian sperm via decreased damage to the sperm. The hypo-osmotic swelling test provided a form of membrane stress test, which is particularly useful when testing the membrane-stabilizing action of antioxidants [36]. In this study, the highest HOST rates were obtained when sperm samples were cryopreserved with 0.50 or 1.00 mM GSH. This may be due to that the antioxidants (GSH) may facilitate the maintaining of post-thawed sperm membrane stability against oxidative stress. The results of the present study revealed that addition of 0.50 or 1.00 mM GSH to the semen extender significantly ($P<0.01$) improve the post-thawing sperm quality including sperm motility, viability, acrosomal and plasma membranes integrity during incubation at 37°C for 3 h. However, there were non-significant differences between the treated and control

samples at the same storage time. Moreover, addition of 0.50 or 1.00 mM GSH can't preserve the post thawing semen quality after 3 h. as well as the control. It appears that the appropriate reason for this is not known.

Sperm DNA integrity appears to be important for correct spermatozoa functioning [37, 38]. The variations in temperature (during cooling, freezing and thawing) can cause detrimental changes in sperm functions and structure (nucleus and membrane) that can impair its fertility capabilities [39, 40]. The present results show that sperm DNA integrity of buffalo semen is impaired during the routine cryopreservation procedure in tris egg yolk glycerol extender (1.50±0.40, 2.30 + 0.62 and 6.80 + 0.56%) for fresh, cooled and frozen thawed semen, respectively. Similar results were reported [41] in bull semen. Cryopreservation of spermatozoa reduces motility and affect DNA integrity [23, 42, 43]. The present investigation reported that addition of glutathione in a dose of 0.50 or 1.00 mM significantly decrease the DNA damage of buffalo spermatozoa compared to untreated semen samples (2.80 + 0.40 vs. 6.80 + 0.56 %). This result may be due to the antioxidants effect of GSH. The addition of antioxidant such as GSH to equine semen [44, 45] has been shown to protect sperm against the harmful effects of ROS. However, the potential role of antioxidants in preventing DNA damage induced by ROS during cryo-preservation had not been studied until now [43]. The ROS released during cooling induce oxidative DNA damage in bovine spermatozoa [38]. In the post thaw buffalo semen samples we observed about 6.80% DNA damaged. This is lower than to the findings reported in stallion [46]**,** bull [47] and boar [48] spermatozoa whereas higher level of damaged DNA was recorded. In the current study, the lower DNA damage observed after GSH treatment suggests the protective effect of GSH on DNA. The most important factors responsible for disruption of condensation and stability of the sperm chromatin after cryopreservation is ROS generation and redoxo balance [49].

The fertility rate is considered to be the best parameter to assess the quality of frozen-thawed semen [50]. On the other hand, laboratory testes can indicate the extent of sperm damage during freezing –thawing.but they cannot accurately predict fertility of spermatozoa. The over all pregnancy rate (50.00% ) recorded in the present study after AI was in agreement with previous studies [51] which recorded a pregnancy rate of 50.40% for frozen- thawed buffalo semen and higher than (42.70, 39.80 and 37.50 % ) in surti buffaloes [52]. In the present study, the pregnancy rate was significantly higher in GSH treated samples than control. The improvement of pregnancy rate may be due to that GSH help maintaining the integrity of normal acrosome [29] and stabilize the plasma lemma of spermatozoa and increase the sperm motility. Also, the improvement in pregnancy rate may be proved by the significant increase in post-thawing sperm motility, viability. Moreover, the glutathione has a protective effect on DNA.The favorable influence of GSH on post- thaw sperm characteristics was a meanable for the improvement in fertility of frozen semen in glutathione treated samples.

In conclusion, addition of 0.50 or 1.00 mM GSH in the preserved liquid buffalo semen extended the keeping quality up to 5 days. Incorporation of 0.50 or 1.00 mM GSH on the semen extender significantly improves the motility, viability, plasma and acrosomal membranes integrity, reduce DNA damage and improve the fertility of frozen thawed buffalo semen.

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