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Effect of glutathione supplementation in extender on quality and fertility of cryopreserved buffalo semen

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ABSTRACT

Glutathione is integral component of cell membrane that has the ability to protect the structural and functional integrity of buffalo spermatozoa during freeze-thawing. The present study was carried out to assess the effect of different concentrations of glutathione on freezability and *in vivo* fertility of buffalo sperm. Twenty four semen ejaculates from four healthy breeding Murrah buffalo bulls were collected using artificial vagina. Qualified ejaculates (≥70% motility and ≥3 mass activity) were diluted with Tris-citric acid extender containing 0.0 (control), 0.5, 1.0, 1.5 and 2.0 mM/ml glutathione at 37°C and cryopreserved following established protocol. The frozen-thawed semen was evaluated for CASA-based sperm kinematic traits, viability, plasma membrane integrity, acrosome integrity, mitochondrial membrane potential and lipid peroxidation (LPO). The sperm total and progressive motility, viability, plasma membrane integrity and mitochondrial membrane potential were higher in the extender containing 0.5 mM/ml as compared to other concentrations of glutathione and control. No difference in the percentage of intact acrosomes was observed in all experimental extenders containing glutathione and control. The malondialdehyde (MDA) production was lower in extender supplemented with glutathione (0.5 mM/ml and 1.0 mM/ml) than in other treatments and control. A total of 60 inseminations were performed with the best evolved extender having glutathione (0.5 mM/ml) and control (0.0 mM/ml, 30 inseminations each). *In vivo* fertility rates were non-significantly higher with extender containing glutathione (0.5 mM/ml; 46.7%) than with control (33.3%). In conclusion, supplementation of 0.5 mM/ml glutathione in extender improved the quality and fertility of cryopreserved buffalo semen.

Keywords: Buffalo bull semen, Cryopreservation, Fertility, Glutathione, LPO, Sperm attributes

Successful freezing of semen along with acceptable fertility rate is the essential requirement to get maximum benefits from artificial insemination. However, owing to cryoinjuries during freeze-thaw procedures, buffalo sperm are alleged to have poor semen quality. One possible cause of poor freezability of buffalo bull semen is inherent susceptibility to free radical damage causing structural changes to sperm membrane (Nasiri *et al.* 2012). Detailed analysis of various additives revealed a need to identify suitable molecules for minimizing the loss due to cryodamage in buffalo spermatozoa. Among various additives, amino acids have been considered to be potent component of semen extenders due to their beneficial effects on semen quality (Bucak *et al.* 2009). Amino acids play an important role in regulation of plasma membrane function, maintenance of sperm viability and fertility during chilling and freezing (Medeiros *et al.* 2002). Further, inclusion of amino acids having antioxidant properties in cryopreservation media improved the quality of semen against reactive oxygen species' (ROS) induced damage (Sarıözkan *et al.* 2009). Glutathione is a naturally occurring tri-peptide that helps in maintaining plasma membrane integrity and works as an antioxidant to protect the bovine sperm cells against ROS (Ansari *et al.* 2014). Moreover, glutathione, cysteine and glycine are major amino acids that contribute 53% of amino acid composition in seminal plasma (Ugur *et al.* 2020). Previous studies in bulls (Ansari *et al.* 2014) and boars (Gadea *et al.* 2004) have shown successful use of glutathione on cryopreserved semen. However, studies in buffalo bulls are sparse and limited. Hence, the present work was planned to evaluate the effect of glutathione supplementation in freezing media on freezability and *in vivo* fertility of buffalo spermatozoa.

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MATERIALS AND METHODS

Semen collection, evaluation and cryopreservation: Twenty four semen ejaculates were collected in the morning hours using artificial vagina from four healthy breeding Murrah buffalo bulls (aged 4-6 years; body weight 680.9±20.0 kg) at different intervals. All bulls were maintained under uniform feeding and management systems at University bull farm and had free access to water throughout the day.

Each ejaculate was divided into five equal fractions and

extended to final concentration of 80×10^6 sperm/ml using Tris-egg yolk semen extender supplemented with different doses of glutathione (catalog no. 158349; Sigma-Aldrich, St. Louis, USA) including 0.0 (control), 0.5, 1.0, 1.5 and 2.0 mM/ml. Ejaculates having initial motility ≥70% and mass activity ≥3 were used in the present study. In brief, the diluted semen of each ejaculate was loaded into 0.25 ml plastic straws (IMV Technologies L'Aigle, France). Thereafter, the straws were allowed to equilibrate at 4°C for 3-4 h in a cold handling cabinet and finally cryopreserved. Following cryopreservation, the post-thaw semen was evaluated within a week. The post-thaw semen of each ejaculate was evaluated for CASA-based sperm motility and kinematics, viability, acrosome integrity, mitochondrial membrane potential, plasma membrane integrity and LPO.

Assessment of sperm motility and kinematics: Immediately after thawing in water bath, 10 µL of post-thaw semen following dilution (1:10) with phosphate buffer solution (PBS) was placed on a pre-warmed disposable glass slide and evaluated (Rijsselaere *et al.* 2003) using a previously calibrated Computer Assisted Semen Analysis (CASA, Androvision, Minitube, Germany). For each semen sample, five fields with at least 150 sperm per field were considered. The CASA software settings were as follows: pixel size (horizontal/vertical)=3.45×3.45 µm, resolution (horizontal/vertical) = 2048×2048 pixels, interface=75 fps, frame rate=USB 3 vision, housing size (L×W×H)=29.3×29×29 mm, lens mount=C-mount, sensor size (optical)=2/3 inch, weight (typical)=80 g, sensor size = 8.45×7.07 mm, sensor type=CMOS, VCL cut-off= 48 µm/s and VSL cut-off=24 µm/s for recording sperm kinetics and motility. The CASA-based motion characteristics recorded in frozen-thawed samples included total motility (TM, %), progressive motility (PM, %), velocity straight line (VSL, µm/s), velocity average path (VAP, µm/s), velocity curvilinear (VCL, µm/s), amplitude of lateral head displacement (ALH, µm/s) and beat cross frequency (BCF, Hz) of the spermatozoa.

Sperm viability: The live sperm count was determined as per the method described by Pintado *et al.* (2000). Briefly, 1.5 µL of Hoechst 33342 (5 µg/ml) – PI (3 µg/ml) stain was mixed to 50 µL of post-thaw semen and incubated at 38°C for 15 min. Then 10 µl of pre-warmed sample was placed on a microscopic slide and examined under 20× magnification using CASA machine (Androvision, Minitube, Germany). At least 200 spermatozoa from each slide were counted in different fields. The spermatozoa which emitted red fluorescence were considered dead and those which emitted blue fluorescence were live.

Plasma membrane integrity: Plasma membrane integrity was examined using hypo-osmotic solution (HOS, 150 mOsmol/L containing 9.9 g D-fructose, 4.9 g Trisodium citrate, DDW up to 1000 ml; Prasad *et al.* 1999). To 100 µl semen, 1.0 ml of hypo-osmotic solution was added. In HOS, the semen was incubated for 1 h at 37°C. Exactly 10 µl of incubated semen from HOS was placed on glass slide and covered with cover slip. The semen was observed at 40× magnification under bright field microscope. A total of 200 spermatozoa were counted in HOS having curled and non-curled tails and the sperm with curled tails were expressed in percentage.

Acrosome integrity: Acrosome integrity was determined according to the method described earlier (Fischer *et al.* 2010). In brief, 27 µl of Hoechst 33342 (5 µg/ml) – FITC-PNA (50 µg/ml) was mixed to 50 µl of frozen-thawed semen and incubated at 38°C for 20 min. Thereafter, 10 µl of pre-warmed sample was placed on a microscopic slide and examined at 20× magnification using triple-band-filter with fluorescence-modules which can read sperm stained in both Hoechst and FITC-PNA simultaneously, characteristic of Androvision. At least 200 spermatozoa from each slide were counted in different fields. The spermatozoa which emitted green fluorescence were considered as damaged acrosomes and those which reflected blue fluorescence were considered as intact acrosomes.

Mitochondrial membrane potential: The mitochondrial membrane potential was determined as per the method described by Pintado *et al.* (2000). To 4 µl of Hoechst 33342-Rhodamin 123 stain, 50 µl of diluted semen was added and incubated at 38°C for 20 min. Thereafter, 10 µl of pre-warmed sample was placed on a microscopic slide and examined at 20× magnification of Androvision. At least 200 spermatozoa from each slide were counted in different fields. The spermatozoa within green box were considered as active mitochondria and those within red box were considered as inactive mitochondria.

Lipid peroxidation in post-thaw semen: The assessment of LPO in post-thaw semen was done through estimation of MDA (Buege and Steven 1978). Briefly, the sperm pellet was suspended in PBS (*p*H 7.2) to obtain a concentration of 20 million/ml. Exactly 1.0 ml of sperm suspension was added into 2 ml of TBA-TCA reagent and heated in boiling water for 1 h. The colour of mixture turned from transparent to yellowish, cooled at room temperature and then centrifuged at 3000 rpm for 10 min. The supernatant was separated and absorbance was read in bio-spectrophotometer (Eppendorf, Hamburg, Germany) at 532 nm. The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5 cm^3) and calculated as per the following:

$$\text{MDA (µmole/20 million sperm/ml)} = \frac{\text{O.D} \times 30}{1.56}$$

In vivo fertility rate: Buffaloes (60) maintained under standard feeding and management systems at university (n=20) and private organized farm (n=40) were enrolled for current fertility trial. All buffaloes (2^{nd} to 5^{th} parity) were apparently healthy and free from physical problems. Two experimental extenders, one having glutathione 0.0 mM/ml (control) and the other having optimal concentration (selected following evaluation of sperm characteristics and LPO from different concentrations) were prepared and used for artificial insemination. Frozen-thawed semen of both extenders was used to inseminate buffaloes (n=30 each). The pregnancy diagnosis was performed on day 60

post-insemination using ultrasonography. The first service pregnancy rate (FSPR) was calculated according to the following:

$$\text{FSPR (\%)} = \frac{\text{Number of buffaloes pregnant after first insemination}}{\text{Total number of first services}} \times 100$$

Statistical analysis: Statistical evaluations were carried out using the GraphPad Prism 8 software. The proportionality data were estimated following arcsine transformation to normalize data. The results were expressed as mean±SEM. One way Analysis of Variance (ANOVA) with the Bonferroni test was used for comparisons of mean values of sperm motion traits, viability, plasma membrane integrity, acrosome membrane integrity, mitochondrial membrane potential and LPO among treatments. The data pertaining to *in vivo* fertility rate was evaluated using Chi-square test. For all analyses, a confidence level of P<0.05 was considered to be significant.

RESULTS AND DISCUSSION

In the present study, CASA-based total motility was higher (P<0.05) in extender containing 0.5 mM/ml glutathione than in other experimental concentrations and control (Table 1). The progressive motility in extender having 0.5 mM/ml glutathione was higher (P<0.05) as compared to that supplemented with 1.5 mM/ml, 2.0 mM/ml and control, and non-significantly (P>0.05) higher than that containing 1.0 mM/ml. These findings are in agreement with the observations of Ogata *et al.* (2021) in crossbred bulls and Shah *et al.* (2017) in Hariana bulls who showed that addition of 0.5 mM/ml glutathione in extender presented higher (P<0.05) sperm motility than in control group. Regarding sperm kinetics, supplementation of 0.5 mM/ml glutathione than other experimental concentrations and control improved (P<0.05) VCL. VAP was higher (P<0.05) in extender supplemented with 0.5 mM/ml and 1.0 mM/ml glutathione as compared to other concentrations. Similar to our study, Perumal *et al.* (2011) in Crossbred Jersey Bulls have also shown significantly higher VCL values in glutathione-treated groups. High VCL and VAP indicated high percentage of sperm showing hyperactive movement which resulted in increased IVF rates and higher proportion of normal sperm morphology (Rashid *et al.* 1998).

Following cryopreservation, higher (P<0.05) proportion of viable spermatozoa were noticed in Tris-egg yolk extender supplemented with 0.5 mM/ml glutathione as compared to other concentrations and control (Table 2). Similar studies in Sahiwal bulls (Ansari *et al.* 2014) and goats (Zou *et al.* 2021) also demonstrated better cell membrane resistance to cryo-damages following supplementation of glutathione at 0.5 mM/ml in comparison to control. The plasma membrane integrity was higher (P<0.05) in semen supplemented with 0.5 mM/ml and 1.0 mM/ml glutathione as compared to other experimental concentrations and control (Table 2). Similarly, Ansari *et al.* (2014) in Sahiwal bulls and Munsi *et al.* (2007) in Holstein bulls recorded higher plasma membrane integrity in Tris-citric egg yolk extender containing 0.5 mM/ml than in control ones. The current study revealed that mitochondrial membrane potential was higher (P<0.05) in semen supplemented with 0.5 mM/ml glutathione as compared to other concentrations and control (Table 2). Limited studies (Gloria *et al.* 2018) in bulls demonstrated higher (P<0.05) mitochondrial membrane

Table 1. Effect of glutathione supplementation in extender on CASA-based sperm motion traits in frozen-thawed buffalo semen (Mean±SEM; n=24 ejaculates).

Parameter	Glutathione (mM/ml)				
	Control (0.0)	0.5	1.0	1.5	2.0
TM (%)	45.6±2.0^{a}	54.2±1.8^{b}	45.9±1.4^{a}	47.0±1.7^{a}	44.0±1.4^{a}
PM (%)	34.9±1.6^{a}	41.4±2.0^{b}	37.7±2.0^{ab}	35.2±1.8^{a}	33.8±1.4^{a}
VSL (µm/s)	82.1±2.4	82.3±2.5	84.3±2.2	81.3±1.8	85.3±3.3
VAP (µm/s)	94.4±3.2^{a}	107.4±3.9^{b}	109.5±3.5^{b}	92.8±2.6^{a}	91.5±2.8^{a}
VCL (µm/s)	130.5±3.5^{a}	165.7±2.9^{b}	150.5±3.3^{c}	135.3±2.1^{a}	139.9±2.7^{a}
ALH (µm)	7.1±0.5	6.2±1.0	7.1±0.6	6.6±0.7	6.8±0.9
BCF (Hz)	39.7±2.8	38.4±3.7	37.2±1.9	38.5±2.4	40.5±2.1

Values with different superscripts within the row differ significantly (P<0.05). TM, Total motility; PM, Progressive motility; VSL, Velocity straight line; VAP, Velocity average path; VCL, Velocity curvilinear; ALH, Amplitude of lateral head displacement; BCF, Beat cross frequency.

Table 2. Effect of glutathione supplementation in extender on sperm function assays in frozen-thawed buffalo semen (Mean±SEM; n=24 ejaculates)

Parameter	Glutathione (mM/ml)				
	Control (0.0)	0.5	1.0	1.5	2.0
Viability (%)	60.2±1.9^{a}	69.4±2.1^{b}	63.5±2.6^{a}	59.8±1.9^{a}	61.6±2.5^{a}
Plasma membrane integrity (%)	44.7±1.4^{a}	50.5±1.5^{b}	50.1±1.2^{b}	46.2±1.4^{a}	45.0±1.6^{a}
Acrosome integrity (%)	61.3±2.4	60.9±1.6	62.2±2.7	59.8±2.0	60.7±2.5
Mitochondrial membrane potential (%)	47.2±1.2^{a}	55.2±2.3^{b}	50.3±1.7^{a}	47.2±1.0^{a}	49.0±2.0^{a}

Mean values with different superscripts within the row differ significantly (P<0.05).

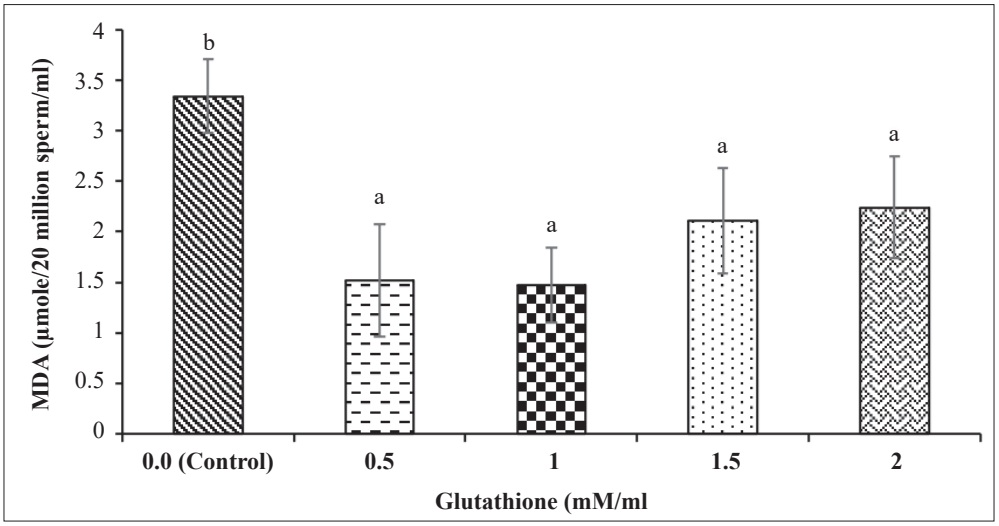

Fig. 1. Effect of glutathione supplementation on MDA levels in frozen-thawed buffalo semen (Mean±SEM; n=24 ejaculates). [a vs b]P<0.05; from corresponding values of control.

potential in Tris citric-egg yolk extender containing 0.5 mM/ml (52.8±1.0%) than in control (36.7±1.7%).

Addition of glutathione to freezing media revealed that MDA levels in all concentrations of glutathione were lower (P<0.05) than control (Fig. 1). Further at 1.0 mM/ml and 0.5 mM/ml, MDA production was lower (P>0.05) in comparison to 1.5 mM/ml and 2.0 mM/ml. Similar studies in bucks also showed that supplementation of glutathione lowered MDA concentrations at 0.5 mM/ml and 1.0 mM/ml compared to control (Sarangi *et al.* 2017).

The first service pregnancy rate (FSPR) was higher (P>0.05) in semen supplemented with the best-evolved glutathione (0.5 mM/ml) concentration (46.7%; 14/30) in comparison to control (33.3%; 10/30). Although, the number of animals inseminated in each group was small, supplementation of glutathione tended to increase (P>0.05) the FSPR (an increase of 13.4%) in comparison to their control counterparts in the present study. Determination of fertility rate gives a most legitimate picture of post-thaw semen quality (Rastegarnia *et al.* 2013). Previous studies (Ansari *et al.* 2014) using Sahiwal bull semen have also shown higher (P<0.05) fertility rates with cryopreserved semen containing 0.5 mM/ml (59%) than in control ones (41%).

Inclusion of glutathione in extender regulates cryoprotective effects on the integrity of axoneme and associated dense fibers in middle piece of spermatozoa that contain mitochondria, generate energy from intracellular stores of ATP resulting in better buffering capacity and subsequently higher sperm motility (Ansari *et al.* 2014). Glutathione in extender might have replaced antecedently lost amino acid concentration during post-thaw stages, reduces ice crystal, increases tolerance limit of osmosis, reinforces sperm plasma membrane, maintains membrane fluidity and subsequently improved sperm cryostability (Abdelrahman *et al.* 2019, Jin *et al.* 2020, Paventi *et al.* 2022). Moreover, improvement in sperm viability has been described to be highly correlated with glutathione content of bull spermatozoa (Stradaioli *et al.* 2007). During storage, excessive generation of ROS by leukocytes is harmful to sperm function (Shi *et al.* 2020). Oxidative stress can disturb the electron transport chain within mitochondria, leading to a decline in the activity of mitochondria (Zhao *et al.* 2019). Supplementation of glutathione in semen extender helps in maintaining the redox balance by preventing excessive ROS production and safeguards active mitochondria, thereby ensuring efficient energy production along with reduced MDA production (Sarangi *et al.* 2017). In the present study, supplementation of glutathione in extender might have stabilized membranes and countered peroxidation thereby leading to increased survival along with efficient movement of post-thaw sperm in female reproductive tract resulting in better conception rates compared to control.

Thus, pre-freeze addition of glutathione at 0.5 mM/ml to extender improved post-thaw semen quality and *in vivo* fertility of buffalo bulls.

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