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Effect of glycine-enriched freezing medium on post-thaw quality and fertility of Murrah buffalo (Bubalus bubalis) bull semen

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Abstract

This study was designed to evaluate the effect of glycine supplementation (0.0, 5.0, 10.0, 15.0, 20.0 and 25.0 mM) in freezing media on post-thaw quality of buffalo bull spermatozoa. Thirty-six semen ejaculates from six breeding Murrah buffalo bulls were collected using artificial vagina. Ejaculates having ≥ 70% sperm motility, ≤ 20% morphological abnormalities and > 500 million/ml concentration were diluted in Tris-egg yolk extender containing selected concentrations of glycine and control and cryopreserved following established protocol. The post-thaw semen was examined for CASA-based sperm motility and kinematics, viability, plasma membrane integrity, acrosome integrity, mitochondrial membrane potential and LPO. Statistical analysis was done using the IBM SPSS 20.0 software and Microsoft excel 2021. The total motility, progressive motility and plasma membrane integrity were higher ($P < 0.05$) in cryodiluent containing 20.0 mM of glycine in comparison to other concentrations and control. Regarding sperm kinematics, VAP was higher ($P < 0.05$) at 20.0 mM, VCL at 20.0 mM and 25.0 mM and VSL at 15.0 mM and 20.0 mM compared to other concentrations and control, respectively. Supplementation of glycine at 20.0 mM and 25.0 mM resulted in higher ($P < 0.05$) proportion of viable sperm. Mitochondrial membrane potential was higher ($P < 0.05$) at 15.0–25.0 mM glycine concentrations compared to remaining concentrations and control. MDA profiles were lower ($P < 0.05$) in all glycine supplemented groups, with lowest levels at 20.0 mM and 25.0 mM than in control. Sixty animals were inseminated using best evolved extender having glycine (20.0 mM) and control (n = 30 each). The conception rates were non-significantly ($P > 0.05$) higher using extender containing glycine than in control (53.3% vs 40%). In conclusion, addition of 20.0 mM glycine in freezing medium improved the sperm characteristics and conception rate of post-thaw Murrah buffalo bull semen.

Introduction

Artificial insemination (AI) using frozen semen is the most extensively employed reproductive technology for genetic improvement, as it allows widespread dissemination of superior germplasm and facilitates organized breeding programs (Singh et al. 2018). However, to maximize the benefits of AI, successful semen cryopreservation accompanied by satisfactory fertility outcomes is essential (Mittal et al. 2019). In India, considerable focus has been given toward advancing buffalo breeding techniques, through semen cryopreservation and AI (Sansone et al. 2000; Singh et al. 2013a). At the same time, buffalo bulls are alleged to have low semen freezability due to cryoinjuries that occur during the freezing and thawing processes. Despite continuous refinement of techniques, nearly 50% of sperm viability is lost during the freeze-thaw procedure (Anzar et al. 2002). During cryopreservation sperm are exposed to cellular and molecular stresses, including cold shock, osmotic stress, membrane lipid phase transitions and oxidative injury induced by reactive oxygen species (ROS) (Mittal et al. 2019; Sharafi et al. 2022). Buffalo sperm are highly susceptible to lipid peroxidation (LPO) due to the abundance of polyunsaturated fatty acids in their membranes and their inherently low antioxidant defense capacity, exhibiting greater sensitivity to oxidative stress compared to cattle spermatozoa (Nair et al. 2006; Bansal and Bilaspuri, 2011). The interaction between membrane lipids and ROS renders these lipids vulnerable to damage, causing structural and functional alterations that lead to reduced fertilizing ability of

spermatozoa (Bisla et al. 2021). To counteract these cryoinjuries, various supplementary agents are incorporated into cryopreservation media to preserve semen quality, primarily by mitigating oxidative stress (Soria-Meneses et al. 2025).

Additives used in semen extenders are broadly classified into membrane-permeating compounds which can freely diffuse across the sperm membrane and non-permeating compounds, which do not cross the cell membrane (Gómez-Fernández et al. 2012; Altyeb et al. 2022). Recently, glycine has emerged as a potent membrane-permeating additive owing to its beneficial effects on semen quality (Peng et al. 2026)., Glycine together with cysteine and glutathione accounts for nearly 53% of the total amino acid content in bovine seminal plasma (Ugur et al. 2020). Glycine is a versatile intracellular cryoprotectant that helps maintain osmotic balance, prevents excessive intracellular ice crystallization and membrane-stabilizing and antioxidant properties (Ahmadi et al. 2019). Moreover, glycine acts as a critical precursor for the synthesis of intracellular glutathione, a major endogenous antioxidant that neutralizes ROS and limits LPO during the freeze-thaw process (Luberda, 2005; Adeoye et al. 2018). Appropriate inclusion of glycine in semen extenders helps maintain sperm morphology and motility by reducing osmotic stress and supporting metabolic stability (Khalili et al. 2010). Although the beneficial effects of glycine supplementation have been documented in several domestic species (Said et al. 2019; Bibi et al. 2022; Nazif et al. 2022), literature regarding its impact on the cryopreservation of buffalo bull semen is scarce and limited. Hence, this study was undertaken to assess the impact of glycine supplementation to the cryodiluent on the post-thaw quality and *in vivo* fertility of buffalo bull semen.

Materials and Methods

Selection of bulls

At bull station, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, six apparently healthy breeding Murrah buffalo bulls having age 5.3 ± 0.2 years (4–6 years) and body weight 732.0 ± 25.4 kg (658–768 kg) were chosen for the study. None of the bulls had any history of physical problems and had good libido and body condition. Each bull was housed in individual pen having covered area (12 x 10 feet) and uncovered area (25 x 10 feet) under loose housing system. All the animals were maintained on regular feeding schedule as per NRC recommendations and management conditions (NRC 2001). The buffalo bulls were fed twice daily (10:30 AM in morning and 3:30 PM in evening) along with *ad libitum* clean drinking water throughout the day. Using artificial vagina, semen from each bull was collected in the morning hours. The study was performed during the months of August through February. During the study period the average relative humidity 68.7 ± 0.8% and daily temperature was 26.4 ± 0.5°C inside the shed.

Semen collection, evaluation, extension and cryopreservation

Thirty-six semen ejaculates from six buffalo bulls (six ejaculates/bull) were collected using an artificial vagina. Only ejaculates having sperm motility ≥ 70% and morphological abnormalities ≤ 20% evaluated subjectively using a phase-contrast microscope and sperm concentration > 500 million/ml assessed objectively with an Accucell spectrophotometer, were included in the experiment. Each ejaculate was diluted to a final concentration of 80×10^6 sperm/ml using a Tris-egg yolk-based extender, divided into six equal portions and supplemented with varying concentrations of glycine including 0.0 mM (control), 5.0 mM, 10.0 mM, 15.0 mM, 20.0 mM and 25.0 mM to determine the most effective concentration of glycine for its cryoprotective properties. Following dilution, the extended semen was equilibrated at 4°C for four hours in a cold handling cabinet. After equilibration, the semen was loaded into 0.25 ml plastic straws and cryopreserved. The straws were exposed to liquid nitrogen vapors at a height of 4 cm for approximately eight minutes and then plunged into liquid nitrogen for storage. For evaluation, the frozen semen was thawed in a water bath at 37°C for 30 seconds. The post-thaw semen of each ejaculate was assessed using CASA-based sperm motility and kinetics, viability, plasma membrane integrity, acrosome integrity, mitochondrial membrane potential (MMP) and lipid peroxidation (LPO) to determine the optimal concentration of glycine.

Estimation of CASA-based sperm motility and kinetic traits

The different sperm motion traits were evaluated using a previously calibrated Computer-Assisted Semen Analysis (CASA, Androvision, Minitube, Germany). The software settings of the CASA used in the present study are according to the manufacturer's 'operating instructions for use' given in their user manual including Pixel size (horizontal/vertical) = 3.45 µm x 3.45 µm, resolution (horizontal/vertical) = 2048 pixels x 2048 pixels, interface = USB 3 Vision, frame rate = 75 fps, lens mount = C-mount, housing size (L x W x H) = 29.3 x 29 x 29 mm, digital input = 1, digital output = 1, weight (typical) = 80 g, power consumption = 3.4 w, sensor type=CMOS, sensor size (optical) = 2/3 inch, sensor size = 8.45 mm x 7.07 mm, VSL cut-off = 24 µm/s, VCL cut-off = 48 µm/s, BCF cut-off = ≥ 20 Hz and ALH cut-off = ≥ 3 µm. Exactly 10 µL of frozen-thawed semen was placed on a pre-warmed disposable glass slide to analyze the CASA based sperm motion traits. In each semen sample, the average of five optical fields of the glass slide were used to count spermatozoa. The following sperm motion characteristics viz. : total motility (TM, %): total motile spermatozoa; progressive motility (PM, %): spermatozoa with progressive motility; velocity curvilinear (VCL, µm/s): the average velocity measured over the actual point-to-point track followed by the cell; velocity straight line (VSL, µm/s): the average velocity measured in a straight line from the beginning to the end of the track; velocity average path (VAP, µm/s): the average velocity of the smoothed cell path; beat cross frequency (BCF, Hz): the frequency of sperm head crossing the average path in either direction; amplitude of lateral head displacement (ALH, µm): the mean width of the head oscillation as the sperm cells swim.

Assessment of sperm attributes in post-thaw semen

Sperm viability

To 1.5 µL of Hoechst 33342 (5 µg/ml) – PI (3 µg/ml) stain was mixed to 50 µL of frozen-thawed semen in an Eppendorf tube (2 ml) at 38°C. The semen stain mixture was incubated in a dark chamber at 38°C for 15 minutes to protect from light. Thereafter, 10 µl of the pre-warmed sample was placed on a microscopic slide and covered with a cover slip. The stained sperm cells were identified under microscope at 20x magnification of Androvision. At least 200 spermatozoa from each slide were counted in different fields. The spermatozoa which emitted red fluorescence were considered dead and those which reflected blue fluorescence were live. Thereafter, module “viability” of Androvision was selected to record the percentage of viable sperm objectively.

Acrosome integrity

Briefly, the frozen semen straw was thawed at 37°C for 30 seconds. Exactly 27 µl of Hoechst 33342 (5 µg/ml) – FITC-PNA (50 µg/ml) stain was mixed to 50 µl of frozen-thawed semen in an Eppendorf tube (2 ml) at 38°C. The semen stain mixture was incubated in a dark chamber at 38°C for 20 minutes to protect from light. Thereafter, 10 µl of pre-warmed sample was placed on a microscopic slide and covered with a cover slip. The stained sperm cells were identified under microscope at 20x magnification of Androvision. At least 200 spermatozoa from each slide were counted in different fields. The spermatozoa within the green box had intact acrosome and those within red box exhibited damaged acrosome. Thereafter, module “acrosome integrity” of Androvision was selected to record the percentage of intact acrosomes objectively.

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 HOS 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 40x 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.

Estimation of mitochondrial membrane potential

To 4 µL of Hoechst 33342–Rhodamine 123 stain, 50 µL of diluted semen was added and incubated at 38°C for 20 min. Thereafter, 10 µL of the pre-warmed sample was placed on a microscopic slide and examined at 20× magnification using AndroVision. At least 200 spermatozoa were counted from each slide in different microscopic fields. Spermatozoa appearing within the green box indicated active mitochondria, whereas those within the red box indicated inactive mitochondria.

Assessment of lipid peroxidation (LPO) in frozen-thawed semen

The assessment of LPO in post-thaw semen was done through estimation of MDA (Buege and Steven 1978). The sperm pellet was suspended in PBS (pH 7.2) to obtain a concentration of 20 million/ml. Exactly 1.0 ml of sperm suspension was added into 2.0 ml of TBA-TCA reagent and heated in the boiling water for 1 h. The color of the solution turned from transparent to yellowish and samples were allowed to cool at room temperature. The samples were centrifuged at 3000 rpm for 10 minutes at room temperature. The supernatant was separated and absorbance was read in bio-spectrophotometer (Eppendorf, Hamburg, Germany) at 535 nm against blank. The MDA concentration was determined by the specific absorbance coefficient (1.56×10^5 cm^3). The calculation of MDA value (µmol/20 million sperm/ml) was done as per the formula given below:

$$\text{MDA (µ mole/20 million sperm/ml)} = \frac{\text{O.D.}\times10^6\times\text{Total volume of reaction mixture (3ml)}}{1.56\times10^5\times\text{Total sample volume (1ml)}}$$

In vivo fertility

The fertility trial was conducted on sixty buffaloes maintained under standard feeding and management systems at university dairy farm (n = 20) and at private organized dairy farm (n = 40) located in peri urban areas of Ludhiana. All the buffaloes (2nd to 5th parity) selected were apparently healthy, calved 60–90 days back and free from reproductive and physical problems, if any. All the buffaloes were inseminated at spontaneous estrus according to the am-pm/ AM-PM/ AM/PM rule (double inseminations at 12 h interval) from December through January. Two experimental extenders, one having glycine 0.0 (control) and the other having most optimal concentration (based on assessment of sperm characteristics) were prepared and cryopreserved as described in earlier section under 2.2. The Frozen-thawed semen of control and glycine treated groups were used to inseminate buffaloes (n = 30 each).

Pregnancy diagnosis

At University dairy farm, the diagnosis of pregnancy was performed on day 60 post-insemination using ultrasound machine (Exago, IMV Technologies, L'Aigle, France) equipped with 5/7.5 MHz interchangeable linear array rectal transducer (ALR, 575 probe). Prior to scanning, the rectum was evacuated by back racking. The acoustic surface of transducer was lubricated with K-Y jelly. The animal was declared pregnant if anechoic amniotic vesicle and hyperechoic fetus appeared on the screen. At private dairy farm, the pregnancy diagnosis was also conducted at day 60 following AI on the basis of per-rectal examination. The conception rate was calculated according to the following method:

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

Statistical analysis

Statistical analysis was carried out using the IBM SPSS software: Statistical version 20 and Microsoft excel 2021. The proportionality data were estimated following arcsine transformation to normalize data. One way Analysis of Variance (ANOVA) was carried out for CASA-based sperm motion traits, plasma membrane integrity, viability, acrosome integrity, membrane integrity, MMP and LPO among treatments and the means were compared with the Duncan Multiple Range Test. The data pertaining to *in vivo* fertility was evaluated using Chi-square test. A confidence level of $P < 0.05$ was considered to be significant for all analyses. The results are expressed as Mean ± SEM.

Results and Discussion

CASA-based sperm motility and kinematics in post-thaw buffalo bull semen

The effect of glycine supplementation on post-thaw CASA-based sperm total motility and progressive motility has been presented in Table 1. The present study revealed that both total motility and progressive motility were higher ($P < 0.05$) in semen incorporated with 20.0 mM than in other experimental concentrations and control. Similarly, inclusion of extender with 25.0 mM glycine exhibited high ($P < 0.05$) total and progressive motility then remaining tested concentrations and control. Amongst different sperm kinematic traits, supplementation of 20.0 mM and 25.0 mM glycine enhanced ($P < 0.05$) curvilinear velocity (VCL) compared to other experimental concentrations and control. Incorporation of 20.0 mM glycine also led to higher ($P < 0.05$) average path velocity (VAP) in comparison to other tested concentrations and control. Further, addition of glycine at 15.0 mM 20.0 mM resulted in higher ($P < 0.05$) straight-line velocity (VSL) than in other concentrations and control. No difference ($P > 0.05$) was observed in beat-cross frequency (BCF) and amplitude of lateral head displacement (ALH) between different concentrations of glycine and control. Motility is considered to be the most important trait for the assessment of fertilizing potential of spermatozoa since it is an important determinant of active metabolism and maintenance of membrane integrity and is the major contributing factor towards successful fertilization (Adeoye et al. 2018). Limited studies (El-Sheshtawy et al. 2008) in buffalo bulls have shown higher total motility in semen extended with glycine (25.0 mM) in comparison to other tested concentrations and control. Further, Said et al. (2019) also reported that addition of 15.0 mM glycine resulted in an increase in post-thaw sperm motility as compared to control in Sumba Ongole bull spermatozoa. In Sahiwal and Achai bull semen, higher progressive motility has been described even at lower concentrations of glycine i.e. 5.0 mM and 10.0 mM respectively (Bibi et al. 2022; Nazif et al. 2022). This variation in optimal concentration may be attributed to species-specific differences in the polyunsaturated fatty acid (PUFA) content of the sperm plasma membrane, which necessitates the

requirement for addition of specific concentration of additives in the extender (Nair et al. 2006; Andrabi et al. 2009). Cryopreservation leads to structural damage of the axoneme, mitochondrial components and plasma membrane in spermatozoa (Awda et al. 2009). Excessive production of reactive oxygen species (ROS) during freeze-thaw procedures further aggravates lipid peroxidation (LPO) and disrupts mitochondrial function, thereby impairing ATP production necessary for flagellar movement (Awda et al. 2009). Khalili et al. (2010) documented that inclusion of glycine in the semen extender exerts a cryoprotective effect by preserving the integrity of the axoneme and the associated dense fibres in the midpiece. These structures contain mitochondria, support ATP production from intracellular reserves, enhance buffering capacity and ultimately improve sperm motility. Glycine plays a role in reducing lactic acid accumulation in extenders and contributes to sperm osmolarity which has a positive role in sperm vitality and motility (Khalili et al. 2010). Moreover, glycine acts as an antioxidant and plays a significant role in the production of antioxidant enzymes that protect spermatozoa against oxidative stressors, thereby preserving motility (Adeoye et al. 2018). During prolonged storage, the nutritive components of semen extenders gradually decline which can lead to a marked reduction in sperm motility (Salamon and Maxwell, 2000). Therefore, pre-freeze inclusion of optimal concentration of suitable exogenous additive (glycine) may help modulate the antioxidant balance of cryopreserved semen and subsequently enhance the post-thaw sperm function and fertility (Said et al. 2019). However, decline in sperm characteristics observed at concentrations exceeding the optimal level can be attributed to the potential toxicity of the amino acid when used in excess. The toxicity of higher amino acid concentrations during the cryopreservation has been recognized in several species including stallion and bull (Amirat et al. 2004; Khlifaoui et al. 2005), buck (Ahmad et al. 2008), ram (Sanchez-Partidata et al. 1992) and human (Foote, 1970). The toxic effect of higher concentration of amino acid(s) is mainly due to their osmotic toxicity and hypertonicity (Khlifaoui et al. 2005). Accordingly, previous studies (Sanchez-Partida et al. 1992; El-Sheshtawy et al. 2008) have shown that lower concentrations (20–70 mM) of glycine manifest their cryoprotecting efficacy on sperm in the presence of glycerol. Similar to our findings, Nazif et al. (2022) in Achai bulls demonstrated improvement in sperm motion traits (VCL, VSL and VAP) following inclusion of glycine (10.0 mM). High VCL and VAP reflected high proportion of sperm showing hyperactive movement which resulted in increased IVF rates and higher percentage of sperm with normal morphology (Hirano et al. 2001). Overall, treatment with glycine (20.0 mM) enhanced sperm velocity traits and thus improved motility.

Table 1
CASA-based sperm motion traits (Mean ± SEM) in post-thaw buffalo bull semen cryopreserved in cryodiluent supplemented with different concentrations of glycine (n = 36 ejaculates)

Parameters	Glycine (mM)					
	0.0 (Control)	**5.0**	**10.0**	**15.0**	**20.0**	**25.0**
TM (%)	51.8 ± 1.7[a]	51.2 ± 1.5[a]	50.3 ± 1.6[a]	50.1 ± 1.7 [a]	61.4 ± 2.0 [c]	56.2 ± 1.5[b]
PM (%)	36.9 ± 1.3 [a]	36.0 ± 1.4 [a]	36.2 ± 1.3 [a]	38.2[a] ± 1.7 [a]	44.9 ± 1.4 [c]	39.9 ± 1.2[b]
VCL (μm/s)	68.4 ± 3.6[a]	65.8 ± 5.0[a]	62.4 ± 3.5[a]	64.5 ± 4.1 [a]	79.1 ± 3.1 [b]	79.6 ± 4.0[b]
VSL (μm/s)	30.7 ± 1.4[a]	30.9 ± 1.2 [a]	28.6 ± 2.3 [a]	34.4 ± 1.5 [b]	36.2 ± 1.6 [b]	25.9 ± 1.6[a]
VAP (μm/s)	39.5 ± 1.7 [ab]	37.3 ± 1.5 [a]	37.7 ± 2.2 [ab]	35.9 ± 1.7 [a]	49.0 ± 2.0 [c]	41.8 ± 1.4 [b]
BCF (Hz)	9.8 ± 0.6	9.1 ± 0.8	9.1 ± 0.7	9.7 ± 0.8	9.0 ± 0.7	8.6 ± 0.8
ALH (μm)	0.98 ± 0.21	0.81 ± 0.12	1.09 ± 0.29	0.88 ± 0.10	0.94 ± 0.11	0.74 ± 0.09
Mean values with different superscripts within the row differ significantly ($p < 0.05$).						

Abbreviations: TM = Total motility; PM = Progressive motility; VCL = Velocity curvilinear; VSL = Velocity straight line; VAP = Velocity average path; BCF = Beat cross frequency; ALH = Amplitude of lateral head displacement

Sperm attributes in post-thaw semen

The proportion of viable sperm were higher ($P < 0.05$) in cryodiluent supplemented with 20.0 mM and 25 mM glycine in comparison to other concentrations and control (Table 2). The sperm plasma membrane must remain intact and viable for ideal sperm function. Addition of 15.0 mM glycine resulted in higher ($P < 0.05$) proportion of viable sperm as compared to control in Sumba Ongole bulls (Said et al. 2019). Similarly, Nazif et al. (2022) reported that the percentage of viable sperm was improved in Achai bull semen supplemented with 10.0 mM glycine than in control. Correspondingly in Moghani rams, inclusion of glycine (15.0 mM) enhanced the viability in comparison to the control group (Khalili et al. 2010). Addition of glycine in the extender might have reduced the ice crystal formation, replaced and/or replenished antecedently lost antioxidants during freeze-thaw stages, increased the tolerance limits of osmosis and subsequently improved sperm cryostability (Khalili et al. 2010; Nazif et al. 2022). Glycine is a positively charged molecule that can electrostatically interact with phosphate groups of sperm plasma membrane phospholipids, thus forming a protective layer on the sperm surface against thermal shock (El-Sheshtawy et al. 2008; Bibi et al. 2022). Moreover, glycine protects sperm viability by scavenging lipid

peroxides since oxidative agents destroys the sperm cell membrane resulting in cellular aging, thus altering semen composition following cryopreservation (Nazif et al. 2022). Said et al. (2019) suggested that glycine uptake by spermatozoa helps in maintaining cellular vitality, stabilize cell membrane proteins thus protecting the sperm cell structure during the freeze-thaw process. Hence, supplementation of glycine to the freezing media under optimal doses (20.0 mM) may have resulted in a higher proportion of viable spermatozoa owing to lesser impairment of the membrane.

Table 2
Sperm attributes (Mean ± SEM) in post-thaw buffalo bull semen supplemented with different concentrations of glycine (n = 36 ejaculates)

Parameters	**Glycine (mM)**					
	Control (0.0)	**5.0**	**10.0**	**15.0**	**20.0**	**25.0**
Viability (%)	50.8 ± 1.2^{a}	52.2 ± 1.2^{a}	50.30 ± 2.7^{a}	53.1 ± 1.6^{a}	62.9 ± 1.5^{b}	61.8 ± 1.6^{b}
Acrosome integrity (%)	67.1 ± 1.6	66.4 ± 2.3	68.8 ± 2.4	66.91 ± 1.6	67.5 ± 1.6	68.3 ± 1.4
Plasma membrane integrity (%)	43.4 ± 1.0^{a}	41.0 ± 2.1^{a}	45.1 ± 1.4^{a}	43.2 ± 1.3^{a}	55.9 ± 1.1^{c}	49.4 ± 1.1^{b}
Mitochondrial membrane potential (%)	41.8 ± 2.0^{a}	45.2 ± 0.9^{b}	46.3 ± 1.3^{b}	55.5 ± 1.5^{c}	54.1 ± 1.5^{c}	55.5 ± 1.6^{c}
Mean values with different superscripts within the row differ significantly ($p < 0.05$).						
Abbreviation: PMI = Plasma membrane integrity, MDA = Malondialdehyde						

Plasma membrane integrity was higher ($P < 0.05$) in semen supplemented at 20.0 mM than in other concentrations and control. Similarly, the proportion of intact plasma membrane was higher ($P < 0.05$) at 25.0 mM than in remaining concentrations and control. Sperm survival is largely dependent on the intact plasma membrane and has been linked with improved fertility *in vivo*. Any injury to the structural integrity of plasma membrane of spermatozoa results in loss of homeostasis and cellular death. The findings of our study are in line with the observations of El-Sheshtawy et al. (2008) in buffalo bulls who recorded higher ($P < 0.05$) percentage of intact plasma membranes in the extender containing 25.0 mM glycine as compared to the control, indicating resistance of the plasma membrane to cryo-damages following inclusion of glycine. Nazif et al. (2022) also observed that higher plasma membrane integrity at 10.0 mM glycine in comparison to the control in Achai bull semen. Excessive ROS production during cryopreservation adversely affects membrane integrity (Awda et al. 2009). Glycine possesses antioxidant properties that show scavenging effects against free radicals and stimulate the production of antioxidant enzymes, thereby enhancing the protection of the plasma membrane against ROS (Nazif et al. 2022). In addition, glycine acts as an organic osmolyte that plays a crucial role in osmoregulation

protects the sperm cell against severe osmotic fluctuations encountered during the freeze-thaw procedure (Khalili et al. 2010). The superior membrane integrity may be attributed to the specific ability of glycine to stabilize the structure of membrane proteins and maintain the lipid-protein organization of the bilayer (El-Sheshtawy et al. 2008). Therefore, a higher proportion of sperm with intact plasma membrane in the extender supplemented with 20.0 mM glycine than in their counterparts may have resulted from lesser membrane damage due to this membrane-stabilizing and antioxidant effect.

The percentage of intact acrosomes did not seem to be affected following addition of glycine since no difference ($P > 0.05$) was noted across all treatments and control. For successful fertilization, the functional and morphological intactness of acrosome is important because spermatozoa having intact acrosome are only able to penetrate the zona pellucida and fuse with oocyte (Buffone et al. 2014). In contrast to our findings, El-Sheshtawy et al. (2008) in buffalo bulls stated that inclusion of glycine to extender at different concentrations (25.0 mM) had a positive effect on acrosome integrity than in control. Similarly, Bibi et al. (2022) also reported that addition of 5.0 mM glycine improved acrosome integrity in Sahiwal bull semen compared to the control. Stresses of freeze-thaw procedures contribute toward impairment of acrosome (Kumar et al. 2019). Glycine, through improved antioxidant activity helps in maintaining the ultrastructure of sperm acrosome membrane and, thus, plays a pivotal role in protection of acrosomes from impairment. Nevertheless, in the current study, supplementation of glycine to the cryodiluent failed to improve the percentage of sperm with intact acrosomes among all treatments in comparison to control.

The mitochondrial membrane potential was similar ($P > 0.05$) in semen supplemented with 15.0–25.0 mM glycine and higher ($P < 0.05$) compared to remaining experimental concentrations and control. The maintenance of active mitochondria is essential for energy production in spermatozoa, regulation of ROS and is linked to sperm capacitation processes, all of which are required for successful fertilization (Alamo et al. 2020). Similar to our study, (Nazif et al. 2022) in Achai bulls recorded higher ($P < 0.05$) mitochondrial membrane potential in extender containing 10.0 mM glycine than in control. The effect of glycine on mitochondrial membrane potential of spermatozoa is linked to its role in mitigating oxidative stress (He and Woods, 2003) which can disturb the electron transport chain within mitochondria, leading to a decline in the activity of mitochondria (Zhao et al. 2019). In addition, glycine helps in maintaining the redox balance by preventing excessive ROS production and safeguards active mitochondria, thereby ensuring efficient energy production (Nazif et al. 2022). The preservation of sperm viability is linked to the maintenance of mitochondrial function (Mehdipour et al. 2025). The higher proportion of viable sperm observed at 20.0 mM in the present study corresponds to improved mitochondrial membrane potential since glycine helps to maintain the functional integrity of mitochondria required for sperm survival (Nazif et al. 2022). Measurement of mitochondrial membrane potential is one of the sensitive parameters for assessing the semen quality (Gualtieri et al. 2021), and hence, variation between different concentrations of glycine and control in the proportion of active mitochondria could be appreciated in present study.

Lipid peroxidation (LPO) in post-thaw semen

The end product of LPO is estimated by the amount of malondialdehyde (MDA). Inclusion of glycine to the extender revealed that MDA levels at all concentrations of glycine were lower ($P < 0.05$) than control (Fig. 1). MDA production was further lower ($P < 0.05$) at 20.0 mM and 25.0 mM concentrations than at 5.0 mM, 10.0 mM and 15.0 mM concentrations. Malondialdehyde (MDA) is considered to be the most potent biomarker of LPO in all cell types including spermatozoa (Kadirvel et al. 2014). Nazif et al. (2022) reported that while they did not measure MDA directly, the total antioxidant capacity (T-AOC) was higher ($P < 0.05$) in Achai bull semen supplemented with 10.0 mM glycine compared to the control. An increase in antioxidant capacity is inversely proportional to LPO, supporting the reduced MDA levels observed in the present study (Nazif et al. 2022). During cryopreservation, addition of glycine reduces the production of ROS which primarily cause LPO, thus preventing oxidative stress (Bibi et al. 2022; Nazif et al. 2022). In the current study, greater reduction ($P < 0.05$) in MDA at 20.0 mM and 25.0 mM concentrations compared to the control, suggested a more profound protective effect of glycine in buffalo semen at these concentrations. Alternatively, Kumar et al. (2019) reported no difference in seminal plasma LPO between glycine-supplemented and control in chicken semen, which might be due to differences in sperm membrane composition and susceptibility to ROS. Glycine shows scavenging effects against free radicals, thus playing an important role in maintaining sperm function through inhibition of LPO (Nazif et al. 2022). As reported earlier, lower increment of MDA could be due to the scavenging action of glycine on free radicals, thus preventing sperm function damage (Khalili et al. 2010). Additionally, amino acids are precursors of intracellular glutathione biosynthesis, which further prevents hydrogen peroxide-mediated loss of sperm motility (Said et al. 2019). In the present study, supplementation of glycine in the freezing media might have lowered MDA production leading to reduced oxidative stress to sperm cells.

In vivo fertility trials

On the basis of the data obtained from various sperm attributes and antioxidant status, the best-evolved glycine concentration for its cryoprotective effect was 20.0 mM. Accordingly, using the most effective concentration of glycine (20.0 mM) and control, 60 inseminations were performed (30 each). The conception rate using post-thaw buffalo semen containing 20.0 mM glycine and control has been presented in Fig. 2. Although, non-significant ($P > 0.05$) the conception rate was higher using semen supplemented with 20.0 mM glycine (53.3%; n = 16/30) in comparison to control (40%; n = 12/30) which may partially be due to small number of animals inseminated in each group and reveal the potential of glycine to enhance fertility outcomes in buffalo. Studies regarding the effect of glycine supplementation on conception rates are not available in literature. Addition of glycine to semen extender tended to increase (by 13.3 percent) conception rate than in their control counterparts in the current study. Determination of fertility rate gives a more legitimate picture of the post-thaw semen quality (Vale et al. 2014). The conception rate in buffalo depends upon semen handling, semen quality, site of semen deposition, season of breeding, insemination technique, fertilization status and time of AI (Dalton et al. 2010). In the current study, efforts were made to minimize the variations due to the above-mentioned factors. During cryopreservation, there is altered influx of calcium, changes in fluidity and damage of plasma membrane damage (Tartaglione and Ritta, 2004). Further, reduction in optimal proportion of suitable additives may also have an impact on other physical properties of sperm membrane including

permeability and temperature (Bansal and Bilaspuri, 2010). In the present study, inclusion of glycine might have counteracted peroxidation, stabilized membranes and decreased calcium uptake, thus, leading to increased survival along with efficient movement of frozen-thawed spermatozoa in female reproductive tract resulting in better conception rates compared to control.

Conclusion

In conclusion, pre-freeze supplementation of glycine (20.0 mM) in cryodiluent improved post-thaw sperm attributes and conception rate as exhibited by better outcomes of cryopreserved semen vis-à-vis fertility.

Abbreviations

TM
Total motility
PM
Progressive motility
VCL
Velocity curvilinear
VSL
Velocity straight line
VAP
Velocity average path
BCF
Beat cross frequency
ALH
Amplitude of lateral head displacement

Declarations

Compliance with ethical standards The Institutional Animal Ethics Committee (IAEC) of the Guru Angad Dev Veterinary and Animal Sciences University approved this study (GADVASU/2025/IAEC/79/13) and is in accordance with the norms of Committee for Control and Supervision of Experiments on Animals (CCSEA), Ministry of Fisheries, Animal Husbandry and Dairying, Department of Animal Husbandry and Dairying, Government of India. All the procedures complied with welfare standards and were conducted under veterinary supervision.

Competing Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality in this experiment.

Data Availability Statement

Data will be made available on request.

Author Contributions

Y. Kumar conducted the experiment. A. K. Singh designed the work and prepared the manuscript. Y. Kumar and G. K. Mavi evaluated sperm characteristics and assessed MDA. Y. Kumar and N. Singh collected, processed and cryopreserved the semen. N. S. Ratta conducted fertility trials and ultrasound scanning. M. Honparkhe performed statistical analysis. P. Singh edited the manuscript.

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Figures

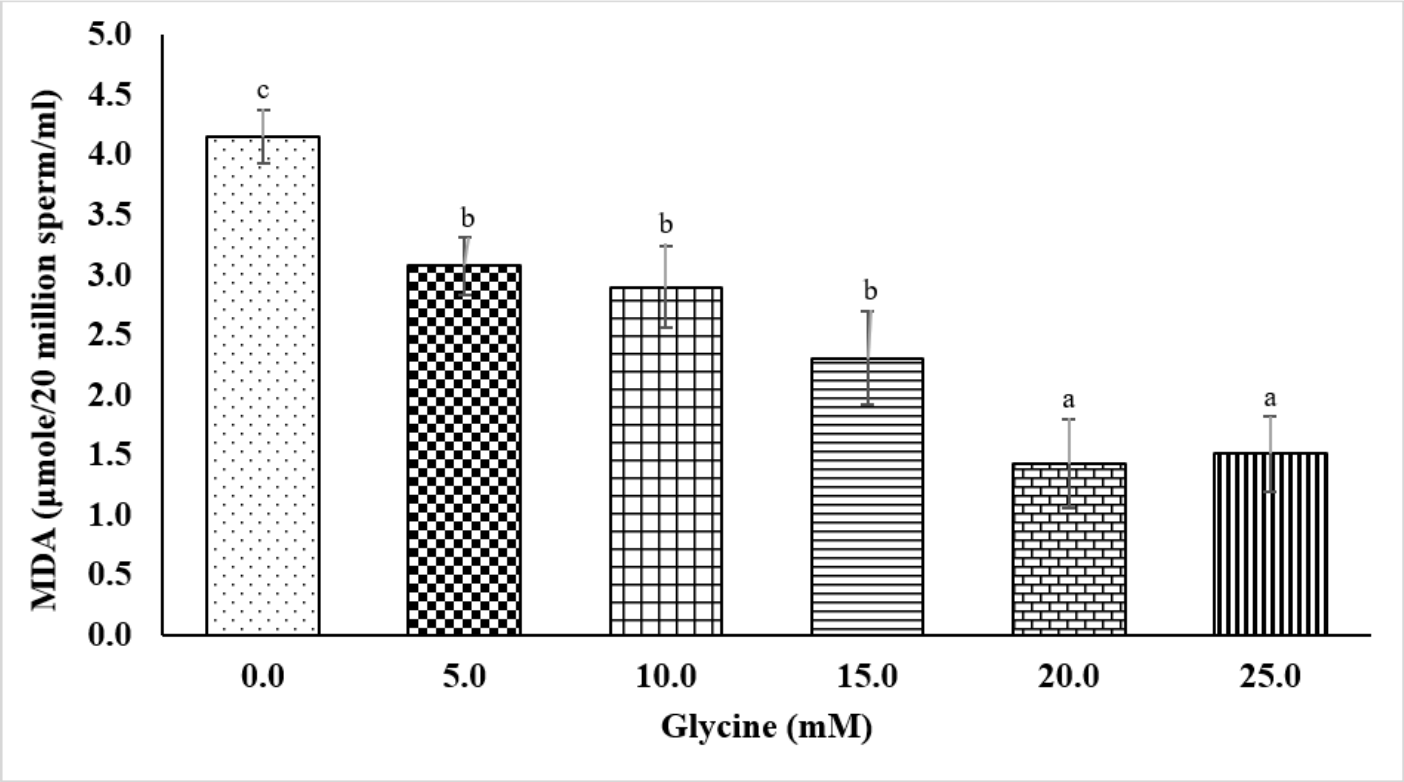


Figure 1

MDA (µmole/20 million sperm/ml) levels in frozen-thawed buffalo bull semen cryopreserved in cryodiluent supplemented with different concentrations of glycine (n=36 ejaculates). Values with different superscripts differ significantly ($p<0.05$).

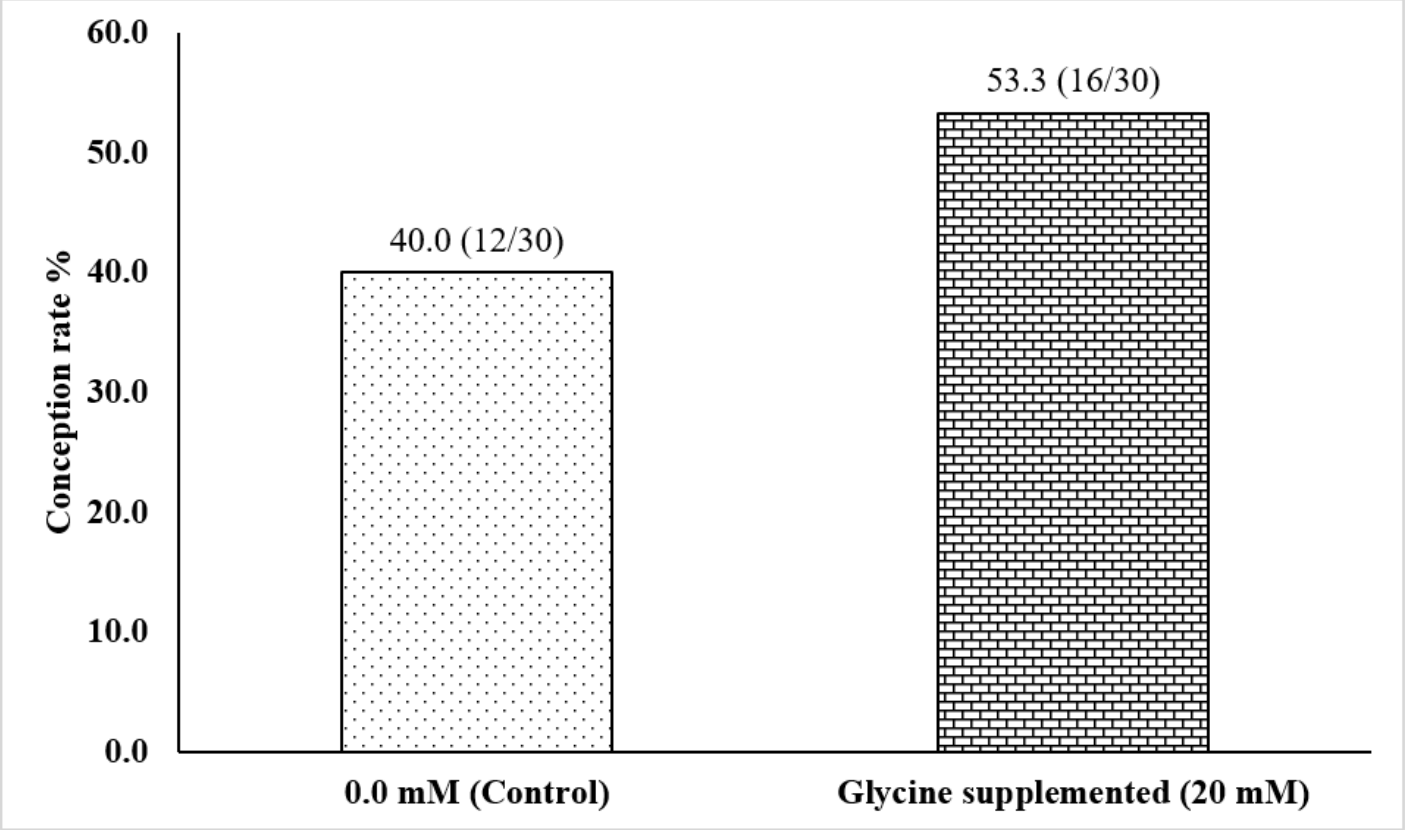


Figure 2

Conception rate using buffalo bull semen cryopreserved in cryodiluent supplemented with glycine (20.0 mM; n=30) and control (n=30)