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In Vitro Supplementation of Glycine Improves Quality of Cryopreserved Bull Semen

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Abstract

Process of cryopreservation induces cell damages due to thermal and osmotic shocks. Addition of amino acids as cryoprotective agents in extender can reduce these damages. Therefore, present study was designed to evaluate the effect of glycine supplementation in extender on freezability of cryopreserved bull semen. For this purpose, three adult Sahiwal bulls were selected for semen collection by artificial vagina having 42°C (three weeks) (replicates; n=18). Collected semen was quickly shifted to laboratory for initial evaluation. Qualified semen ejaculates (>60% motility, >1mL volume, >0.5billion/mL conc.) were diluted in *tris* extender having 0 (control), 5, 10, 15 and 20mM of glycine. Semen was cooled to 4°C in 2 hours and kept for 4 hours for equilibration. Semen was filled in French straws at 4°C and kept in liquid nitrogen container. Highest percentages of post thaw sperm progressive motility and acrosome integrity were observed in extender containing 5mM of glycine compared to other treatments and control ($P<0.05$). However, sperm viability, chromatin integrity and plasma membrane integrity remained same. In conclusion, glycine addition at 5mM in extender improves quality of cryopreserved bull semen.

Keywords: Glycine, amino acid, cryopreservation, bull semen

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1. INTRODUCTION

During cryopreservation semen quality is adversely affected due to possible consequences like, formation of intracellular ice crystals, thermal and osmotic shock [1] and damage to sperm membranes [2]. Mammalian sperm membranes are prone to lipid peroxidation [3] due to occurrence of relatively high level of polyunsaturated fatty acids and antioxidant content in cytoplasm, resulting in disintegration of sperm membranes during freeze-thaw steps of cryopreservation [4]. Reactive oxygen species play vital role in sperm hyperactivation, acrosome reaction, capacitation and zona binding [5], but increased production of ROS during cryopreservation is harmful for sperm viability [6]. Therefore, various antioxidants have been added in extender to protect sperm membranes from cryodamages [7].

Amino acids have shielding effects on sperm membranes against cryodamages [8, 9]. Amino acids are also speculated to keep calcium-dependent ATPase of sarcoplasmic reticulum [10] and some enzymes [11] during the freezing process. Moreover, addition of amino acids reduces membrane lesion and lipid peroxidation and plasmolysis by their membrane stabilizing property [12, 13]. Addition of cysteine or glycine in extender enhances quality of cryopreserved ram sperm [14]. As glycine has protective effect in extender on the cryopreservation of mammalian spermatozoa, therefore, the present study was planned to evaluate the effect of glycine supplementation in extender on freezability of bull sperm.

2. MATERIALS AND METHODS

2.1. Preparation of Extender

Tris-citric acid was used as a buffer (pH=7, 320mOsmol Kg^{-1}) consisted of 3.0 g *tris*–(hydroxymethyl)-aminomethane and 1.56 g citric acid (Research Organics, USA; Fisher Scientific, respectivelys) in 73 mL distilled water was used. The pH and osmotic pressure of the buffer were 7.0 and 320 mOsmol Kg^{-1} respectively. Fructose 0.2% wt/vol (Scharlau, Spain), glycerol 7% (Riedel-deHaen, Germany), egg yolk of fresh hen 20% v/v, antibiotics combination [(1000 IU/mL) benzyl penicillin and (1000 ug/mL) streptomycin sulphate: available as Sinbiotic® (China)] were added to above buffer. Five semen extenders were supplemented with glycine at the rate of 5, 10, 15, and 20 mM (experimental extenders). Extender without glycine addition was considered as control.

2.2. Ethical Statement

The present research work was conducted with the approval of the ethical committee of university of Pir Mehr Ali Shah, Arid Agriculture University Rawalpindi.

2.3. Semen Collection

Three adult Sahiwal bulls of similar age (7-8 years) and known fertility kept at semen production unit Qadirabad, Sahiwal, Pakistan, were selected for this study. Two consecutive ejaculates (6 ejaculates/bull/experiment) were collected per bull once a week for 3 weeks by artificial vagina at 42°C. The ejaculates were immediately transferred to the laboratory for initial evaluation. Percentage of motile sperm was observed by phase contrast microscope (X400) [15]. Neubauer haemocytometer was used to assess the concentration of sperm. The qualified semen samples (>1 ml vol., >60% motility and >0.5 billion sperm/mL conc.) were processed further. Experimental extenders were prepared with this pooled semen at 37°C by supplementing 0, 5, 10, 15 and 20mM of glycine.

2.4. Semen dilution and freezing

Dilution of semen aliquots was done with one of the five experimental media (37°C) at rate of 50 million sperm/mL, semen was allowed to cool at 4°C in 2 h and kept for 4 hours for equilibration. Semen was filled in French straws at 4°C and kept in liquid nitrogen container (-196°C). Thawing was performed at 37°C (30 sec.) after 24 hours of cryopreservation for evaluation of semen quality.

2.5. Post-thaw sperm functional assays

2.5.1 Motility of forward progressive sperm

Percent sperm progressive motility was assessed using a phase contrast microscope (X 200; Olympus BX 20) after placing a drop (5 mL) of the semen sample on a pre-warmed (37°C) glass slide.

***2.5.2* Sperm Membrane Integrity**

Sperm membrane integrity was assessed by Hypo-osmotic (HOS) swelling test [16]. HOS solution was made by 0.73 g of sodium citrate and 1.35 g of fructose in 100 mL distilled water (osmotic pressure ~190 mOsmol Kg^{-1}). For assessment, 50 µl of frozen-thawed semen and 500 µl of HOS solution (37°C) was mixed and incubated for 45 min. (37°C). After incubation, 10 µL of semen was placed on a slide and viewed by phase contrast microscope (X400). A total of 200 sperm per treatment per replicate were counted and characterized on the basis of swelling and coiling of sperm tail. Swollen tails of sperm were indicated as

intact, biochemically active sperm membranes, while unswollen tails were indicated as disrupted, inactive, non-functional sperm membrane.

2.5.3 Sperm viability

Dual staining procedure was used to evaluate sperm viability as practiced by Qadeer et al. [16]. For assessment, 5µL semen was mixed with 5 µL of trypan blue solution (MP Biomedicals, Eschwege, Germany) and air dried for 10 minutes. Fixative formaldehyde-neutral red was used to fix the slides (5 minutes), and after rinsing slides were stained (4 hr) with Giemsa stain (7.5%). Air-dried slides were mounted with Canada Balsam and observed under a microscope. A total of 200 sperm per treatment per replication were counted. Sperm that are transparent with bright acrosome were characterized as viable while sperm with dark blue distinction and blur acrosome were characterized as non-viable

2.5.4 Sperm acrosome integrity

Sperm acrosome integrity was examined by mixing 100 µL of semen sample in 500µL of formal citrate (Tris sodium citrate dehydrate (2.9 g) + 37% formaldehyde (1 mL) in 100 mL of distilled water. Sperm heads with clear demarcation were considered to have intact acrosomes while sperms with blur ends were considered to have damaged acrosomes.

2.5.5 Sperm chromatin integrity

To assess sperm chromatin integrity, air dried smears of semen samples were fixed in 96% ethanol-acetone (1:1) at 4°C for 30min and hydrolyzed with 4N HCl at 25°C for 10-30 min. Smears were suspended in distilled water, three times for two minutes each. The slides were stained with toluidine blue in McIlvaine buffer (sodium citrate-phosphate) for 10min. Samples were air dried and mounted with Canada Balsam. A total of two hundred spermatozoa per experimental extender per replicate were evaluated in at least five different fields under a light microscope at 1000X. Toluidine blue stain penetrates in sperm having damaged chromatin staining from dark blue to purple while spermatozoa having intact chromatin were stained light blue.

2.6. Statistical analysis

The effect of various concentrations of glycine supplementation in extender on post-thaw quality of cryopreserved bull sperm was statistically analyzed using analysis of variance in a randomized complete block design using MSTAT-C® (Ver. 1.42; Michigan State University, East Lansing, MI, USA). When F-ratio was found significant ($P < 0.05$), the least significant difference test was used to compare the treatment means.

3. RESULTS AND DISCUSSION

The data on the effect of glycine addition in the extender on the quality of frozen-thawed bull semen are shown in Table 1. Acrosome integrity and sperm motility were improved in extender ($P<0.05$) with 5mM of glycine compared to other treatments and control. However, glycine supplementation in extender didn't affect plasma membrane integrity, viability, and chromatin integrity of cryopreserved bull sperm.

Table 1. Effect of extender supplementation of glycine on post-thaw quality of cryopreserved bull semen.

Glycine (mM)	Sperm progressive motility (%)	Sperm plasma membrane integrity (%)	Sperm acrosome integrity (%)	Sperm viability (%)
0	41.4±2.4^{bc}	57.0±0.5	74.6±0.33^{ab}	43.2±1.0
5	53.3±2.5^{a}	56.2±3.8	78.3±2.6^{a}	43.7±1.2
10	45.0±5.1^{b}	55.9±1.8	72.1±3.2^{bc}	42.4±0.9
15	43.3±4.4^{bc}	55.4±1.8	72.7±3.4^{ab}	42.7±1.4
20	38.9±2.4^{c}	50.6± 2.8	66.8±4.6^{c}	41.2±0.5

Values having dissimilar superscripts within the same column differ significantly ($p < 0.05$). The total no. of semen ejaculates were eighteen (3 replicates per each of three bulls; 2 ejaculates per replication).

Mammalian sperm plasma membrane is more susceptible to ROS-induced oxidative stress during freezing due to the high content of polyunsaturated fatty acids in sperm membranes [12, 17]. Lipid peroxidation of sperm membranes severely affect the functional integrity of sperm membranes [18]. This effect can be mitigated by supplementation of amino acids in semen extender [12].

The addition of glycine in *tris* extender improves freeze-thaw characteristics, such as progressive motility, acrosome intactness, viability, and membrane functionality of ram sperm [14]. The results of the current study are in line with the previous studies suggesting that glycine supplementation improved post thaw motility in ram [19], stallion [8] and goat [20]. A reverse relation in post thaw sperm motility and glycine concentration was observed in present study, which is corroborated in cynomolgus monkey [21] and buffalo semen [22]. Cryopreservation process intensifies the production of toxic ROS, which cause sperm lipid peroxidation, resulting in reduced sperm motility [23]. The probable reason for improvement in sperm motility by glycine supplementation might be due to its cryoprotective effect that can be deduced from their ability to form a sheet resulting from the force of attraction between cationic amino acids and anionic phosphate group of phospholipids, this binding may have a supportive role in sperm vitality [24, 20]. However, decreased sperm motility by increased glycine concentration might have been due to the toxic effect of the higher amino acid concentrations attributable to their harmful osmotic effects [8].

Present study suggested that sperm plasma membrane integrity didn't differ significantly in extenders containing glycine compared to control, contrary to the reports in cynomolgus monkey [21], ram [19], stallion [8, 25], goat [20] and buffalo bull sperms [22]. This anomaly could be attributed to species difference and glycine concentration. It is obvious to mention that at highest amino acid concentration lowest post thaw plasma membrane integrity was shown which was also observed in stallion [25], cynomolgus monkey [21] and in buffalo [22].

Acrosome reaction is vital for the process of fertilization and it relies on the presence of intact acrosome. Improvement in post thaw acrosomal integrity by supplementation of glycine in extender has been reported in goats [20] and buffalo. It is pertinent to mention that in previous studies the minimum concentration of glycine i.e., 40mM in goat [20] and 25mM in buffalo bull [22] gave better results, which is in line with the present studies where better results were obtained with minimum concentration of glycine i.e., 5mM in terms of acrosome integrity. It is clear that glycine supplementation in extender improved sperm acrosome integrity but the exact mechanism is not known.

Cryopreservation induces physico-chemical damages which leads to reduced sperm viability and fertilizing ability [26]. Khalili *et al.* (2010) suggested that glycine supplementation of *tris* extender improved post thaw sperm viability in Moghani rams [14]. However, in present study glycine supplementation in extender didn't affect viability and chromatin integrity of cryopreserved bull semen. In contrary to our findings, glycine addition improved post thaw sperm viability in ram [19], stallion [8, 25] and goat sperms [20]. This difference can be attributed to species specific resistance to cryoinjuries and requirement of a particular concentration of glycine to compensate ROS mediated loss in sperm viability.

4. CONCLUSIONS

In present study, glycine supplementation in extender didn't affect plasma membrane integrity, chromatin integrity and viability of cryopreserved bull sperm, however, addition of 5mM glycine was most advantageous to improve progressive motility and acrosomal integrity of cryopreserved bull sperm. This effect may be attributed to difference in polyunsaturated fatty acids content and the relevant difference in susceptibility of different species to ROS mediated attack [4]. It is concluded that glycine might be employed as a non-toxic and non-permeating antioxidant additive in bull semen extender.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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