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Glutathione-S-transferase: Role in buffalo (*Bubalus bubalis*) sperm capacitation and cryopreservation

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ABSTRACT

In this study, glutathione-S-transferase Mu3 (GST) has been reported to play an important role in sperm capacitation, acrosome reaction, and fertilization. The freshly ejaculated buffalo spermatozoa were *in vitro* capacitated using heparin (10 μg/mL) or cryopreserved in egg yolk citrate extender. Glutathione-S-transferase was identified and characterized in terms of their isozymic forms, tyrosine phosphorylation, and immunolocalization patterns in cryopreserved buffalo spermatozoa in comparison with freshly ejaculated and *in vitro* capacitated spermatozoa. Two-dimensional gel electrophoresis, immunoblot, immunocytochemistry, and enzyme activity analyses were done to characterize GST in this study. Five and eight isozymic forms of GST were detected in cryopreserved and capacitated spermatozoa, respectively. Differential tyrosine phosphorylation of these enzymes was observed in cryopreserved and capacitated spermatozoa. The tyrosine phosphorylation of this enzyme involved cAMP protein kinase-A dependent and extracellular signal-regulated kinase independent pathways during *in vitro* capacitation of the spermatozoa. Differential immunolocalization patterns of GST were observed in freshly ejaculated, capacitated, and cryopreserved spermatozoa. Glutathione-S-transferase Mu3 enzyme activity was found to be significantly ($P < 0.05$) different in freshly ejaculated, capacitated, and cryopreserved spermatozoa. Activity of GST was significantly ($P < 0.05$) increased with the progression of capacitation. The cryopreserved spermatozoa showed significantly ($P < 0.05$) greater enzyme activity compared with fresh spermatozoa and was equal to 2-hour capacitated spermatozoa. The cryopreserved spermatozoa showed significant ($P < 0.05$) loss of GST enzyme protein. Tyrosine phosphorylated GST showed significantly ($P < 0.05$) greater activity compared with their dephosphorylated forms. The information generated in this study can be used to understand the molecular mechanism of the effects of GST on capacitation. Regulation of GST during sperm cryopreservation could be a good target to improve fertility of cryopreserved spermatozoa for their use in assisted reproductive technologies.

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1. Introduction

Products generated by reactive oxygen species (ROS) or metabolites of drugs and chemicals that induce stress must be eliminated from a cell to prevent cell damage. Detoxification of products of oxidative stress and electrophilic compounds is carried out by enzymes called glutathione-S-transferases (GSTs; Enzyme Commission number 2.5.1.18) that constitute the phase II detoxification system in cells, and are encoded by multiple genes in mammals [1]. Studies have demonstrated the presence of immunoreactive and enzymatically active GSTs on the goat sperm surface that serve as oocyte binding proteins [2–6]. Although sperm surface GSTs have been shown to be enzymatically active, the mechanism how it protects spermatozoa is not clear. Compared with somatic cells, spermatozoa are extremely susceptible to damage by ROS and ROS-generated products

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because of the preponderance of oxidation-prone unsaturated fatty acids in their plasma membrane [7].

Aravinda, et al. [2], Gopalakrishnan and Shaha [3], and Hemachand and Shaha [6] showed the presence of GSTs on goat spermatozoa, where it functions as detoxifying enzymes. They also reported that the goat spermatozoa were unable to fertilize oocytes when treated with anti-GST antibodies. Although it was evident from studies that binding of anti-GST antibodies on specific sites of sperm surface GSTs could prevent fertilization, the precise role of sperm GSTs in this process was not clear. Hemachand and Shaha [6] presented clear evidence that GSTs of 24 kDa are present on mature cauda epididymal sperm plasma membrane, and are attached to it by peripheral noncovalent interactions. Moreover, sperm plasma membrane–associated GST activity has been reported to be significantly greater than that of plasma membrane from somatic cells such as brain cells, spleenocytes, ventriculocytes, and hepatocytes [8]. Two GST isoforms, namely GST Mu and GST Pi are present on the sperm plasma membrane, and are capable of binding to the zona pellucida.

The ejaculated spermatozoa are fertilization incompetent and acquire the ability to fertilize the oocyte only after a maturation process in the female genital tract known as "capacitation". The process of capacitation prepares the spermatozoa to undergo acrosome reaction without which there would be no fertilization. Hence, capacitation is the key event in reproduction. Bailey, et al. [9] reported that capacitation-like changes occurs in cryopreserved spermatozoa of bovine, porcine, equine, and hamster, and it decreases the functional life of spermatozoa leading to reduced fertility.

Spermatozoa lack transcriptional and translational activity therefore posttranslational modifications like protein tyrosine phosphorylation regulate important sperm functions like capacitation and acrosome reaction leading to fertilization. Recently, it has been reported that protein tyrosine phosphorylation is an index of sperm capacitation [10]. Gali and Atreja [11] reported a number of tyrosine phosphorylated protins and identified those proteins using two-dimensional (2D) PAGE matrix-assisted laser desorption/ionization (MALDI)-Mass spectrometry/Mass spectrometry during *in vitro* capacitation and cryopreservation of buffalo spermatozoa. Various report also shows that, tyrosine phosphorylated GST play important role in human, mouse and hamster sperm capacitation. The role of GST warrant studies in buffalo sperm capacitation and cryopreservation. Therefore, the present study was designed to identify and characterize GST in terms of its isozymic forms, tyrosine phosphorylation, immunolocalization patterns, and enzyme activity in cryopreserved buffalo spermatozoa in comparison with freshly ejaculated and *in vitro*-capacitated spermatozoa. This will be a step further in understanding the molecular mechanism of sperm capacitation and improvement of the fertility of cryopreserved buffalo spermatozoa.

2. Materials and methods

2.1. Chemicals

Heparin, BSA (fraction V), lysophosphatidylcholine, sodium orthovanadate, protease inhibitor cocktail, goat anti-mouse immunoglobulin G–horseradish peroxidase (IgG–HRP) conjugate, x-ray films (Kodak), x-ray film developer and fixer (Kodak), and GST assay Kit were procured from Sigma-Aldrich (Sigma Life Science, St. Louis, MO, USA). Polyvinylidene difluoride membrane, monoclonal anti-phosphotyrosine antibody (Clone 4G10), and enhanced chemiluminescent reagent (Immobilon Western chemiluminescent HRP substrate) were procured from Millipore (Billerica, MA, USA). Monoclonal anti-GST antibody was procured from Santa Cruz Biotech Inc. All chemicals of 2D, protein molecular weight markers were obtained from Bio-Rad Pvt. Ltd. Qualitative filter papers (Grade-1) and chromatography paper (3 mm) were procured from Whatman Asia Pacific Pvt. Ltd., India. All other chemicals used in this study were of analytical grade and purchased from local suppliers.

2.2. Semen collection, cryopreservation, and in vitro capacitation

Buffalo (*Bubulas bubalis*) bulls of 3 to 5 years of age, were housed at Artificial Breeding Research Centre, National Dairy Research Institute, Karnal, India, under uniform, standard, nutritional conditions, were used in this study. Semen was collected from trained buffalo bulls, twice a week, at an interval of 4 days. Before semen collection, bulls were allowed 2 hours of exercise in bull exerciser. All bulls used in the present study had a field fertility rate of more than 45% when frozen semen was used. A total number of six ejaculates were collected using an artificial vagina (IMV, L'Aigle cedex, France) maintained at 41 °C from six individual bulls chosen at random. Semen volume, sperm mass motility, and individual motility were evaluated immediately after semen collection. Ejaculates showing a score of 3 or more mass activity and more than 80% progressive motile sperm were used in the study. Each ejaculate was split into two aliquots; then one aliquot was used for *in vitro* sperm capacitation and the other was cryopreserved.

2.2.1. Semen cryopreservation

Semen was extended to a final concentration of approximately 80×10^6 cells per mL in tris-based egg yolk extender (274 mM tris, 87 mM citric acid, 43 mM glucose, 1,000,000 IU/L benzyl penicillin and 750,000 IU/L streptomycin 6.4% glycerol, 20% egg yolk). Extended samples were aspirated into small-sized (0.25 mL) French straws, sealed using a sealing machine (IMV), and equilibrated at 4 °C for 4 hours. After equilibration, the straws were frozen in liquid nitrogen vapor, 5 cm above liquid nitrogen, for 10 minutes, and then the straws were plunged into liquid nitrogen for storage.

After storage for 3 to 4 weeks, frozen straws were thawed at 37 °C for 30 seconds in a water bath. Thawed spermatozoa were washed in modified Tyrode's Hepes-buffered medium (sp-TALPH, with 1 mg polyvinyl alcohol per mL, pH 7.40), and used for the study of different sperm parameters [12].

2.2.2. In vitro capacitation of spermatozoa

Another semen aliquot was immediately used for *in vitro* sperm capacitation. This semen was centrifuged at 275 $\times g$

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