Effect of glutathione on membrane integrity of bovine spermatozoa during cryopreservation of extended semen in tris based dilutor

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Abstract

The present investigation was aimed to study the effect of Glutathione on membrane integrity of Hariana bull spermatozoa through Hypo-Osmotic Swelling Test (HOST) during the different stages of semen preservation; after dilution, pre-freeze and post thaw, using Tris based dilutors. After the primary evaluation like progressive motility more than 70% and sperm concentration more than 600 millions spermatozoa/ml of collected ejaculates (N=24) of bulls were used for cryopreservation. The samples were extended in a Glycerolated Egg Yolk Tris (GEYT) Extender upto 80 millions sperm /ml. Addition of various concentrations 0.0 mM (Control), 0.5 mM (T1) & 1.0 mM (T2) of Glutathione was done in the diluted semen. Semen was frozen in liquid nitrogen vapour in French Mini Straw. The plasma membrane integrity of spermatozoa was evaluated through HOST. A significant difference(P<0.05) was observed between control and treatment groups for mean percentage of HOST reacted spermatozoa after dilution of semen with the mean of 76.30±0.38, 85.00±0.41 and 79.83±0.49 percent in control, T1 and T2 groups respectively. Similar trend was also observed at pre-freeze stage in control, T1 and T2 groups with the mean of 67.47±0.34, 77.14±0.83 and 72.72±0.48 percent respectively. The percentage of HOST reactive spermatozoa was significantly (P<0.05) higher in treatment groups at post-thaw stage with mean value of 53.15±0.61, 65.33±0.53 and 59.84±0.58 in control, T1 and T2 groups respectively. Addition of 0.05 mM (T1) Glutathione was found significantly different between the two treatments and more beneficial in terms of membrane integrity compared to 0 mM (control) and 1.0 mM (T2) in all the stages of (after dilution, pre-freezing and following post-thaw) semen preservation. There is no any significant difference between the bulls in term of HOST reactive spermatozoa.

Keywords: Glutathione, hariana bull spermatozoa, membrane integrity, HOST, tris

Introduction

The genetic improvement of livestock species and disease control are of fundamental importance to the success of a sustainable agri-food industry. In this sense, artificial insemination (AI) is arguably the most important tool contributing to the advancement of modern animal production. Successful semen cryopreservation enhances these advantages of AI over natural breeding. It is generally accepted that the consequences of sperm cryoinjury caused by the cryopreservation procedure are impaired transport and poor survival in the female reproductive tract (Salamon and Maxwell, 1995) [10]. The sperm plasma membrane is the primary site of damage induced by cryopreservation (Hammerstedt et al., 1990, Parks and Graham, 1992 and Watson, 1995) [11, 20, 32]. Cryomicroscopic examination of ram sperm loaded with a marker for membrane integrity revealed that exposure to low temperatures followed by warming differentially affected the plasma membranes specially the principal-piece, midpiece, and head (Holt and North, 1994) [11] which is mainly depends on the structural stability of the plasma membrane (de Leeuw et al., 1993) [11]. Reactive oxygen species (ROS) play an important role in fertility/ infertility, when the balance between ROS production and detoxification by antioxidants is disrupted, an excess of ROS creates oxidative stress, ROS such as H$_2$O$_2$ are known to arrest motility and block oxidative metabolism in sperm. Variety of additives like anti-oxidants, membrane stabilizers, motility enhancers and chelating agents have been used to protect spermatozoa from deleterious effects of cryopreservation and for improving freezability and fertility of bull semen. Inclusion of natural antioxidants such as alpha-tocopherol and ascorbate had a protective effect on metabolic activity and cellular viability of cryopreserved bovine (O’Flaherty et al., 1997) [19].
Effect of antioxidant such as iodixanol reduces the capacitance change in changes in the cryopreserved buffalo bull spermatozoa (Yadav et al., 2017) 133.

Glutathione, a tripeptide thiol (γ glutamylcysteinylglycine), is the major non-protein sulphhydryl compound in mammalian cells plays a prominent role in detoxification and antioxidation of exogenous and endogenous compounds, as well as maintaining the intracellular redox status. The sulphhydryl group (SH) of glutathione confers its protective action against oxidative damage. Glutathione exists in two forms: the reduced form (GSH) and the oxidized form (GSSG). The protective action of glutathione against reactive oxygen species (ROS) is facilitated by the interactions with its associated enzymes, such as glutathione peroxidase and glutathione reductase (Storey et al., 1997) 28. Oxidative stress to sperm cell results into lipid peroxidation of plasmalemma, irreversible loss of motility, leakage of intracellular enzymes and damage of the chromatin (Aitken et al., 1997). Glutathione (GSH) addition in bovine semen extender resulted in a decrease of the lipid peroxidation levels during freezing by its scavenging action and plays an important role in the protection against damage produced by oxidants, electrophiles and free radicals owing to its ability to react directly with hydrogen peroxide and superoxide anion, hydroxyl and alkoxyl radicals by its free sulphhydryl groups (Eskiocak et al., 2005; Perumal et al., 2008) 9, 23.

Biochemically active membrane is required in sperm cell for the many biological functions like capacitation, acrosome reaction, binding of the spermatozoa to the egg surface etc (Jeyendran et al., 1984 and Chan et al., 1985) 18, 5. The Hypo Osmotic Swelling Test (HOST) is based on the principle that when the sperm cells are subjected to a hypo-osmotic solution, the cells with intact membranes take up water apparently without a significant enlargement of their area, thus forcing the flexible motor apparatus of the tail to bend and coil (Drevius and Ericksson, 1966) 8. The swelling of sperm tail in a hypo-osmotic solution is due to the transfer of water across the membrane, which is a sign of membrane integrity and normal functional activity. Bending, shortening and / or coiling of the sperm tail is the response that could be observed. Viable sperm in a hypotonic solution has been shown to develop bent and coiled tails (Anderson, 1945, Pursley and Herman, 1950) 5, 29, whereas dead sperm had straight tails associated with cell lysis. Therefore, it was hypothesized that the ability of the sperm to swell in hypotonic solution indicates its membrane integrity and normal function activity (Takahashi et al., 1990) 29. HOST has a high correlation (0.80 in cattle and 0.93 in buffalo) with fertility.

Material methods
Present study was conducted on Hariana bulls of the age group between 5.5 – 6.5 years and weighing between 450-500 kg body weight, reared at the University Instructional Livestock Farm Complex (ILFC), College of Veterinary Sciences and Animal Husbandry, U.P. Pandit Deen Dayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura which is situated in a semiarid zone of Northern India, in the state of Uttar Pradesh. semen collection was made biweekly from each bull with the help of Artificial Vagina (AV) on dummy animal. Collected ejaculates were observed for colour and consistency by direct visualization with naked eyes. pH of the fresh semen was measured by using pH paper. The mass motility was rated according to the vigourous wave motion on grade scale of 0 to 5 and ejaculates having morethan +3.5 mass motility were taken for the futher semen processing. Following evaluation of fresh semen the samples were extended into three parts with three different combination of dilutors i.e. GEYT (Gycerolated Egg Yolk Tris) with no addition of Glutathione (control), GEYT with 0.5mM Glutathione (T1) and GEYT with 1.0mM Glutathione (T2).The first part considered as control while the other two parts contain 0.5mM Glutathione (T1) and 1.0mM Glutathione (T2) were considered as treatment 1 and 2, respectively. These samples were then processed for cryopreservation and thawed thereafter.

Hypo Osmotic Swelling Test (HOST) for sperm membrane integrity

Preparation of Hypo-osmotic solution- The hypo-osmotic swelling test is performed according to the methods described by Correa and Zavos (1994) 10. Hypo-osmotic solutions of 150 mOsmol/litre was prepared after adding the 7.35 gm Sodium citrate, 13.51 gm fructose and double distilled water up to 1000 ml. A pinch of Nigrosin was added in both HOST group and control solution to provide the background colour.

Procedure of HOST: 990 microlitre HOST solution (maintained at 37 °C for 5 min before use) was added to 10 microlitre extended semen and incubated at 37 °C for one hour. Following incubation, a drop of well mixed solution was taken on a clean glass slide and covered with a cover slip allowed 5 min to settle down. Sperm tail curling is recorded as an effect of swelling due to influx of water (Fig: 1a to 1d). A total of 200 spermatozoa were counted in different fields at 40X magnification under phase contrast microscope. These spermatozoa were classified in four different classes according to presence of following swelling pattern; No swelling- no membrane reaction (HOST non- reactive), Swelling of the tip of the tail (HOST reactive), Different type of hair pin like swelling or swelling of mid-piece (HOST reactive), Complete tail coiling (HOST reactive) (Takahashi et al., 1990) 29. The percentage HOST reacted spermatozoa was calculated by dividing the number of reacted cells (curled tails/swelled spermatozoa) from total spermatozoa counted and multiplying the figure by 100. Actual Percentage of HOST reacted spermatozoa were obtained after deduction of number of spermatozoa swollen/curled tail in the control sample (extended semen without HOST solution) keeping the same number of total spermatozoa during counting.

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\text{HOST Reacted Spermatozoa (%) = } \frac{\text{Number of curled tail/swollen spermatozoa}}{\text{Total number of spermatozoa counted}} \times 100
\]

Results
The mean percentage of HOST reactive spermatozoa following the dilution of semen in the GEYT, was significantly higher (P<0.05) (ANOVA & DMRT) among the treatment groups (T1 & T2) as compared to control group and the value was 76.30±0.38, 85.00±0.41 and 79.83±0.49 in control (0mM), T1 (0.5 mM) and T2 (1.0 mM) groups respectively. At pre-freeze stage the mean percentage of HOST reactive spermatozoa was also significantly (P<0.05) (ANOVA & DMRT) higher in treatment groups (T1 & T2) as compared to control group and it was 67.47±0.34, 77.14±0.83 and 72.72±0.48 percent in the control, T1 and T2 group respectively. Similarly, at post-thaw the mean percentage of HOST reactive spermatozoa was significantly (P<0.05)
Membrane integrity is important for spermatozoa while 3mM was found non positive correlation of spermatozoa (Barratt et al., 2015).

The addition of antioxidant like Glutathione might have protective action rendering the better livability percentage in extended Ram semen (Uysal et al., 2007) [10]. The mean progressive motility of spermatozoa in control and Glutathione(GSH) (0.5 mM) treated semen on day 2 post-thaw, had significantly better sperm motility (56.3%) than 0.0 mM (with 55.4% sperm motility) or 1.0 mM (with 52.9% sperm motility) treated samples (Munsi et al., 2007) [18]. In a study on bull semen in liquid state using egg yolk citrate extender glutathione was tested at concentrations of 0.5, 1.0, 2.0 and 3.0 mM (Munsi et al., 2007) [18] and found significantly higher sperm motility at 0.5, 1.0 and 2.0mM added Glutathione while 3mM was found non-beneficial. Sperm acrosomal integrity was conserved letter with 0.5mM of glutathione for five days. Therefore, 0.5mM of glutathione was recommended for use in extender for storage of bull semen in egg yolk-citrate extender. In our study the 0.5 mM Glutathione was found better membrane integrity of spermatozoa as compare to control (0 mM), which is similar to Munsi et al., (2007) [18].

The assessment of the membrane may be a useful indicator of the fertilizing ability of spermatozoa (Barratt et al., 1989 and Avery et al., 1990) [14, 3]. Membrane integrity is important for sperm metabolism, motility, capacitation, acrosome reaction and the binding of spermatozoa to the egg surface (Keel and Webster, 1990) [16]. A significant positive correlation of HOST positive sperm with mass motility, initial progressive motility, live count, total intact acrosome and sperm concentration were recorded by Prasad et al., (1999) [24] and Srivastava and Kumar (2006) [27]. Goswami (2006) [10] and Kale (1995) [15] reported the mean percentage of HOST reacted sperm was 69.66 in crossbred goats. HOST reacted sperm was found 54.02 percent for Sahiwal bulls Keshava et al., (2007) [21] who recorded 79.3 ± 3.7 percent HOST positive spermatozoa after dilution of semen. In relation to this Perumal (2008) [25] also stated that addition of 0.5 mM Glutathione in the bull semen makes 71.29 ± 0.26 percent HOST reactive spermatozoa at post thaw stage. Addition of other antioxidant such as BHT (phenolic antioxidant) at 1.0 mM concentration increases the membrane integrity, sperm viability and acrosomal integrity of spermatozoa during cryopreservation of Hariana bull semen (Patel et al., 2015, 2016) [21]. Our findings (Table1.0) are also has similarity to Patel et al., (2015) [21], that anioxidant improves the membrane integrity of bovine spermatozoa.

### Table 1: Mean percentage of HOST reactive spermatozoa in the semen of Hariana bulls extended in GEYT with Glutathione supplementation during different stages (after dilution & pre-freeze) of cryopreservation and following post-thaw (Mean±SEM, N=24/Group)

<table>
<thead>
<tr>
<th>Stage of Semen Preservation/Groups</th>
<th>Control Group (0 mM)</th>
<th>Treatment 1 (0.5 mM)</th>
<th>Treatment 2 (0.5 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>After Dilution</td>
<td>76.30±0.38 (73.12-79.54)</td>
<td>85.00±0.41 (79.41-88.56)</td>
<td>79.83±0.49 (73.50-83.67)</td>
</tr>
<tr>
<td>Pre-freeze Stage</td>
<td>67.47±0.34 (63.86-70.00)</td>
<td>77.14±0.83 (62.19-82.08)</td>
<td>72.72±0.48 (68.31-77.50)</td>
</tr>
<tr>
<td>Post-thaw stage</td>
<td>53.15±0.61 (48.00-58.30)</td>
<td>65.33±0.53 (59.00-70.00)</td>
<td>59.84±0.58 (55.12-65.00)</td>
</tr>
</tbody>
</table>

Means with different superscripts (a, b, c) differed significantly.

**Fig 1:** Photograph showing Hypo osmotic swelling test (HOS Test) reactive and non reactive spermatozoa. (Hypo osmotic solution 150 mosm/lit. Magnification: 40x) (a) to (d): Various degree’s of HOST reactive spermatozoa. (e): HOST non-reactive spermatozoa.
Summary and Conclusion
Addition of 0.05 mM (T1) Glutathione in the extender during cryopreservation of Hariana bull semen was found significantly different and more beneficial in terms of membrane integrity compare to 0.0 mM (control) and 1.0 mM (T2) in all the stages of (after dilution, pre-freezing and following thawing) semen preservation. Thus, addition of 0.5 mM Glutathione in the bovine semen improves post-thaw membrane integrity of spermatozoa.

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References