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The Pharma Innovation

ISSN (E): 2277- 7695
ISSN (P): 2349-8242
NAAS Rating: 5.03
TPI 2019; 8(6): 734-738

www.thepharmajournal.com
Received: 10-04-2019
Accepted: 12-05-2019

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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 intigrity 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 spermatozaoa 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 aslo observed at pre-freez 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 signinificantly different between the two treatments and more benificial 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 signifcant difference between the bulls in term of HOST reactive spermatozoa.

Keywords: Glutathione, hariana bull spermatozoa, membrane intrigity, 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) [26]. 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) [13] which is mainly depends on the structural stability of the plasma membrane (de Leeuw *et al.*, 1993) [7].

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_2O_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 alfa-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 capacitation like changes in the cryopreseved buffalo bull spermatozoa (Yadav *et al.,* 2017) [33].

Glutathione, a tripeptide thiol (γ glutamylcysteinylglycine), is the major non-protein sulphydryl 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 sulphydryl 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 sulphydryl 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) [14, 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) [2, 25], 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 motality 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 Osmaotic Swelling Test (HOST) for sperm membrane intigrity

Preparation of Hypo-osmotic solution- The hypo-osmotic swelling test is performed according to the methods described by Correa and Zavos (1994) [6]. Hypo-osmotic solutions of 150 mOsmol/litre was prepared after adding the 7.35 gm Sodium citrate, 13.51 gm fructose and double distillted water upto 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 ºC for 5 min before use) was added to 10 microlitre extended semen and incubated at 37 ºC 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/sweeled 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 spermazoa during counting.

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

Results

The mean percentage of HOST reactive spermatozoa following the dilution of semen in the GEYT, was significantly higher (P<0.05) (ANOVA & DMRT) in the treatment gropus (T1 & T2) as compare 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-freez 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 repectively. Similarily, at post-thaw the mean percentage of HOST reactive spermatozoa was significantly (P<0.05)

(ANOVA & DMRT) higher in treatment groups T1 and T2 as compare to control group and it was 53.15±0.61, 65.33±0.53 & 59.84±0.58 in control and treatment groups T1 and T2 group respectively. Comparison (unpaired't'-test) between the bulls for control and treatment groups did not revealed any significant difference at any stage of semen preservation (Table1.0).

Discussion

Routine semen evaluation has certain limitations for comprehensive prediction of fertility of bull semen. The HOST highlights the permeability of sperm membrane to Hypo osmotic solution and the projection of higher value is a valid indication of intact membrane and sample with higher value is regarded as potent for establishing pregnancy. The comparatively significant ($P<0.05$) value after dilution in treatment group 1 (T1; 0.5 mM) with respect to control group (0 mM) and treatment group 2 (T2; 0.5 mM) for HOST is attributed to incorporation of antioxidant (Glutathione). This indicates that the Glutathione have capacity to maintain the membrane integrity. The effect of Glutathione was also significantly better compared to controls however, dose appears to be toxic.

The addition of antioxidant like Glutathione might have protective action rendering the better livability percentage in extended Ram semen (Uysal *et al.,* 2007) [30]. 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 intigrity 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) [4, 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 (1996) [17]. The present finding (Table1.0) is comparable with Uysal *et al*. (2007) [31] who recorded 79.3 ± 3.7 percent HOST positive spermatozoa after dilution of semen. In relation to this Perumal (2008) [23] 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 intigrity, sperm viability and acrosomal intigrity 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 improoves the membrane intigrity 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-free) of cryopreservation and following post-thaw (Mean±SEM, N=24/Group)

Stage of Semen Preservation/Groups	Control Group (0 mM)	Treatment 1 (0.5 mM)	Treatment 2 (0.5 mM)
After Dilution	76.30^{c}±0.38 (73.12-79.54)	85.00^{a} ±0.41 (79.41-88.56)	79.83^{b} ±0.49 (73.50-83.67)
Pre-freez Stage	67.47^{c} ±0.34 (63.86-70.00)	77.14^{a} ±0.83 (62.19-82.08)	72.72^{b} ±0.48 (68.31-77.50)
Post-thaw stage	53.15^{c} ±0.61 (48.00-58.30)	65.33^{a} ±0.53 (59.00-70.00)	59.84^{b} ±0.58 (55.12-65.00)

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

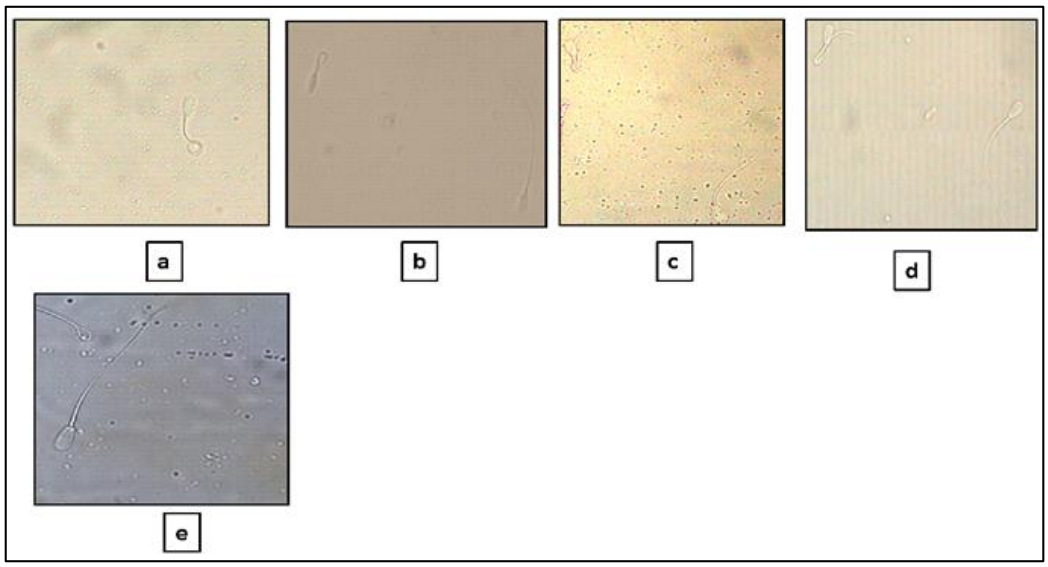

Fig 1: Photograph showing Hypo osmotic swelling test (HOS Test) reactive and non reactive spermatozoa. (Hypo osmotic solution 150 mosm/lit. Magnification: 40x) (a) to (d): Various degree's of HOST reactive spermatozoa. (e) : HOST non-reactive spermatozoa.

Summary and Conclusion

Addition of 0.05 mM (T1) Glutathione in the extender during cryopreservation of Hariana bull semen was found signinificantly different and more benificial 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 intigrity of sprematozoa.

Acknowledgement

I am very thankful to U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalya Evam Go Anusandhan Sansthan, Mathura (U.P.) for providing the all the necessary facilities which were required for conducting this piece of research.

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