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Effect of glutathione on viability and progressive motility of Hariana bull spermatozoa during cryopreservation in semi-arid region

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Abstract
The present study was conducted to evaluate the effect of Glutathione, an anti-oxidant, on the sperm viability and progressive moltility of bovine bull spermatozoa at different stages of semen preservation (After dilution, pre-freeze) and subsequently at post thaw. Following evaluation of fresh semen and those which confirmed the standard as stated vide supra, the semen sample of each bull were split into three equal parts and extended with three different combination of dilutors i.e. Glycerolated Egg Yolk Tris (GEYT) (control), GEYT with 0.5mM Glutathione (T1) and GEYT with 1.0mM Glutathione (T2). These samples were processed for cryopreservation and thawed thereafter. The mean percentage of sperm viability differes significantly ($P<0.05$) between treatment and control group at post-thaw and it was 56.60±0.69, 69.71±0.33 and 64.24±0.52 percent in control, T1 and T2 respectively. Mean percentage of post thaw progressive motility also differed significantly ($P<0.05$) between treatment and control group and it was 47.79±0.65, 60.08±0.64 and 55.21±0.65 percent in control, T1 and T2 respectively. Comparison (Unpaired't' test) between the bulls did not revealed any significant difference.

Keywords: viability, progressive motility, bovine bull spermatozoa, glutathione

1. Introduction
Successful semen cryopreservation enhances the advantages of Artificial Insemination (AI) over natural breeding. For agriculturally important animals, semen cryopreservation is an established industry worldwide, particularly for dairy cattle. Effective semen cryopreservation is a big task because a large number of sperm are apparently infertile following freezing and thawing (Shannon and Vishwanath, 1995) [45].

Livability is the one of major factor for assessment of semen quality. During cryopreservation the spermatozoa are exposed to a foreign diluting media as well as to low and very low temperature. Death might occur due to release of toxic substances, ultra-low exposure, enzymatic leakage, medium of preservation, degree of sperm permeability, aging effect of sperm and individual variation (Salisbury *et al.,* 1979) [42]. It is believed that sperm viability is reduced upto 50% after the freeze-thawing process (Hammerstedt *et al.,* 1990; Watson, 1995; Shannon and Vishwanath, 1995) [19, 54, 45]. Pant *et al.,* (2002) [32] reported that semen with more than 30 per cent initial dead spermatozoa is not good for preservation.

Facilitates sperm transport through the female reproductive tract and is essential for the fertilization. Rapid progressive, sluggish progressive, non-progressive and immotile Oscillatory motility is normally seen in aged sperms, near to death. Rao and Rao (1975) [38], Gupta *et al.,* (1990) [17], Deshmukh *et al.,* (1992) [12], Veerapandian *et al.,* (1992) [53], Rao *et al.,* (1996) [37], Ulfina and Raina (2003) [50] and other workers has estimated the sperm progressive motility in various indigenous cattle breeds. Sagdeo *et al.,* (1990) [41] concluded that post-thaw motility is the main criteria for assessing the freezability of semen. Mohanty (1999) [29] reported that semen having higher initial motility has a higher post-thaw motility**.** The post-thaw motility is significantly correlated ($r = 0.6772$; $P = 0.033$) with *in vivo* fertility of bovine frozen semen (Gillan *et al.,* 2005) [15]. Usually the progressive motility of a good semen sample should range between > 50 to 90 per cent. A relation between initial progressive motility and freezability has been reported (Saxena and Tripathi, 1979) [43].

Cryopreservation of mammalian semen reduces the motility and accelerates the production of ROS molecules through lipid peroxidation levels beyond the physiological levels

(Rasul *et al.,* 2001; Chattergee and Gagnon, 2001; Kadirvel *et al.,* 2009; Bailey *et al.,* 2000) [39, 11, 21, 4]. Sperm motility decreases after cryopreservation along with the total antioxidant potential of the semen (Kumar *et al.,* 2011) [24]. It has been observed that levels of ROS in mammalian semen negatively correlated with the motility (Kadirvel *et al.,* 2009) [21]. The ROS molecules like H_2O_2 reduced the motility of buffalo and bull spermatozoa *in vitro* (Belodeau *et al.,* 2001 and Garg *et al.,* 2009) [6].

The use of antioxidant in extender is recommended to reduce the cryodamage to spermatozoa (Andrabi, 2008) [1]. It is known that all the extenders belonging to third generation like AndroMed (Minitube, Germany), Triladyl (Minitube, Germany), Bioxcell (IMV, France) are supplemented with antioxidant. The antioxidant potential of the mammalian semen is not enough to protect the spermatozoa during cryopreservation against oxidative stress. Therefore, in extender for improving the quality of bovine, caprine, canine, equine, human, swine and buffalo spermatozoa in frozen and liquid state different antioxidants were used viz; Vitamin C (Ball *et al.,* 2001) [5], Vitamin E (Ball *et al.,* 2001; Andrabi *et al.,* 2008) [5, 1], Superoxide dismutase (El-Sisy *et al.,* 2008) [13], Glutathione (Uysal and Bucak, 2007; Buck *et al.,* 2007; Ansari *et al.,* 2010) [51, 10, 2], Iodixanol (Yadav *et al.,* 2016) [55], BHT (Patel *et al.,* 2016) [34] etc.

Bull semen diluents with Glutathione improve post thaw motility and normal acrosome by reducing the enzyme leakage and might be due to limited production of lipid peroxides (Kumar, 2007) [25]. Lipid peroxidation of the spermatozoa negatively correlated with plasmalemma integrity and progressive motility (Kasimanickam *et al.,* 2007) [22]. The addition of antioxidant like Glutathione might have protective action rendering better livability per cent in respective groups (Uysal *et al.,* 2007) [52]. The mean progressive motility of spermatozoa in control and GSH-treated semen had significantly better sperm motility (Munsi *et al.,* 2007) [31]. Glutathione protect the semen quality when exposed to artificial oxidative stress induced by hydrogen peroxide in frozen thawed semen (Belodeau *et al.,* 2001, Bucak *et al.,* 2007) [6, 10].

2. Material and Methods

The present investigation was conducted on Bovine Bull (Hariana Breed) of an about 5.5 – 6.5 year age group having 450-500 kg body weight. The location of study was semiarid zone of Northern India at U.P. Pandit Deen Dayal Upadhayaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anusandhan Sansthan, Mathura (Uttar Pradesh), India. Following evaluation of fresh semen the samples (N=24) were extended into three parts with three different combination of dilutors i.e. GEYT (Gycerolated Egg Yolk Tris) with no Glutathione (control; 0.0mM), GEYT with 0.5mM Glutathione (Treatment-1; T1) and GEYT with 1.0mM Glutathione (Treatment-2; T2). All the samples were processed for cryopreservation after 4 hrs of equilibrium period at 4^0C under the liquid nitrogen vapour. Post-thaw evaluation was done after the thawing of semen straw at 37^0C for 45 sec.

2.1 Viability of spermatozoa

A method described by Bloom (1950) [9] and Hancock (1951) [20] was followed for the demontration of viable spermatozoa. Dead spermatozoa could be differentiated by their ability to get stained by Eosin dye. The live spermatozoa, which are alive at the time of staining, remain colourless since they were impermeable to the Eosin stain. Nigrosin provided a blue-black background**.**

i) Preparation of stain

Eosin-Nigrosin stain was prepared by the method described by Bloom (1950) [9] and Hancock (1951) [20]. The Eosin-Nigrosin stain contains Eosin-Y (Water Soluble) 100mg, Nigrosin (Water Soluble) 500mg, Sodium Citrate buffer (2.9%, pH=6.8) upto 10 ml. The mixture was kept overnight and on the next day filtered through quality filters paper (Whatman filter paper no. 40) and stored in a dark and sealed glass bottle. Before use, the staining solution was brought to room temperature. Fresh stain was prepared every 15 days to prevent artifacts.

ii) Staining technique

Semen samples were kept at 32-34°C and before analysis one drop of semen sample was mixed with 2 to 3 drops of Eosin-Nigrosin stain on a clean glass slide at 32-34^0C using warm stage. It was kept for 3 min. A smear was made from the mixture on a clean and grease free glass slide. It was dried in air and examined under the bright field 100× oil immersion objectives of phase contrast microscope. About 200 sperms were counted. Sperms that were white (unstained) classified as live (Fig: 1, a) and those that showed any pink or red colouration (Fig: 1, b) were classified as dead. Per cent live spermatozoa were calculated by devinding the numbers of viable spermatozoa from total numbers of spermatozoa counted multiply by 100.

2.2 Progressive motility

The progressive motility of the spermatozoa was observed under high power phase objective lens (40 X) on a thermostatically controlled stage maintained at 37°C. A small drop of diluted (1:100) semen was put on a clean grease free slide and covered with a cover slip. The slide was examined to observe vigorously motile spermatozoa exhibiting progressive path. The progressive motility of spermatozoa was then calculated by devinding the numbers of progrssively motile spermatozoa from total numbers of spermatozoa counted multiply by 100.

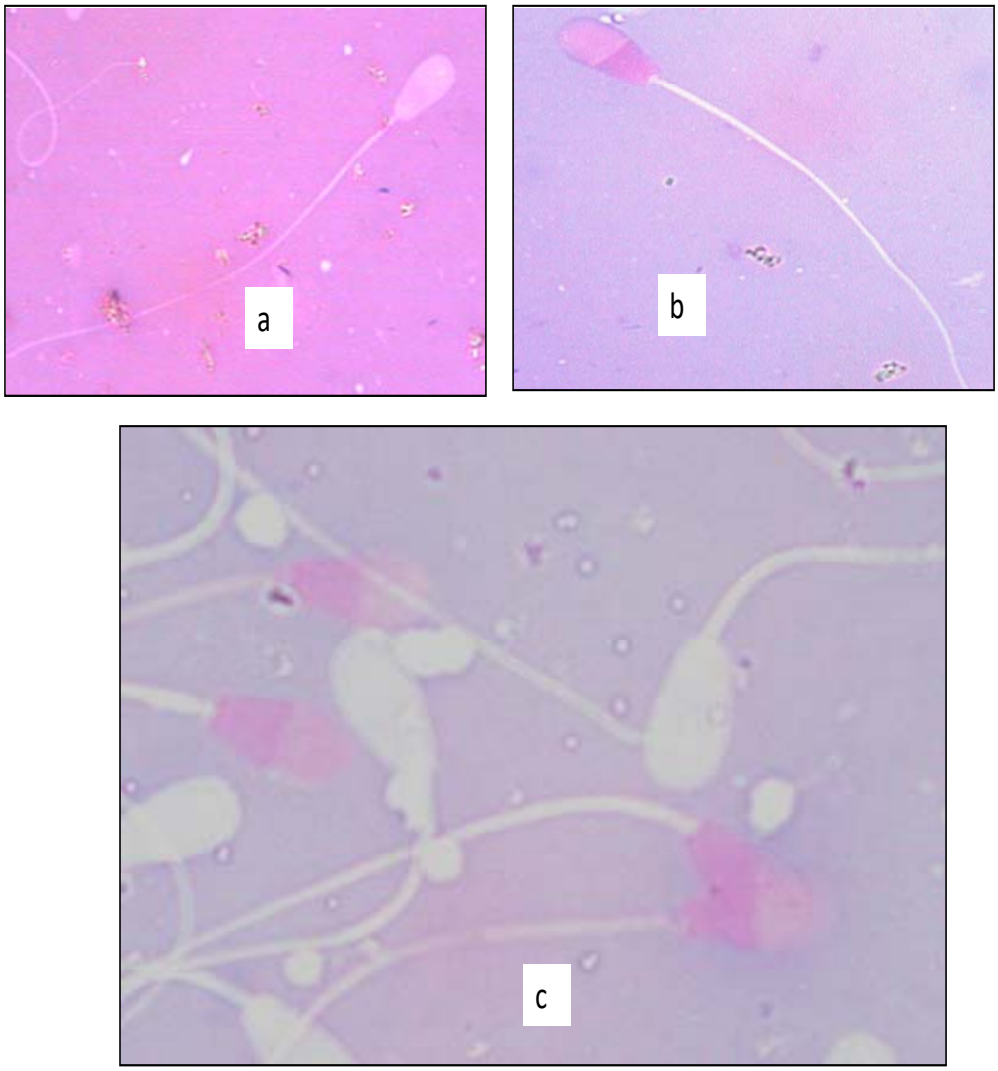

Fig 1: Photograph showing Live and Dead Spermatozoa (Eosin–Nigrosin stain, Magnification: 100X).
(a) Live spermatozoa (non-eosinophilic, **(b)** Dead spermatozoa (partially eosinophilic), **(c)** Live and Dead spermatozoa

3. Result

3.1 Viability of spermatozoa

The mean percentage of live spermatozoa after dilution in control and treatment groups T1 and T2 were 80.35±0.30, 88.73±0.36 and 84.37±0.37 respectively and statistically (ANOVA & DMRT) it was significantly (P<0.05) higher in treatment groups T1 and T2 as compared to control group. At pre-freezing stage the mean percentage of live spermatozoa was significantly (P<0.05) higher in treatment groups T1 and T2 as compared to control group and it was 71.33±0.39, 81.70±0.43 and 76.88±0.40 in control and treatment groups T1 and T2 respectively. The two treatment groups T1 and T2 also differed significantly (P<0.05). After post-thaw the mean percentage of live spermatozoa in control and treatment groups T1 and T2 was 56.60±0.69, 69.71±0.33 & 64.24±0.52 respectively and it was significantly (P<0.05) higher in treatment groups T1 and T2 as compared to control group. Further, the treatment groups T1 and T2 also differed significantly (P<0.05). Further, the treatment groups T1 and T2 also differed significantly (P<0.05) at all stages of semen preservation and at post-thaw also. Comparison (Unpaired 't' test) between the bulls revealed significant (t=0.190) difference in treatment 2 only after dilution, in other stages no any significant difference was observed (Table: 1).

Table 1: Viability of spermatozoa in cryopreserved Hariana Bull semen supplemented with glutathione (Percent Mean ± SEM, N=24)

Percent Viability of Spermatozoa			
Stage of Semen Preservation	**Control (0.0mM)**	**Treatment 1 (0.5mM)**	**Treatment 2 (1.0mM)**
After Dilution	80.35 ^{c} ±0.30 (78.26-83.11)	88.73 ^{a} ±0.36 (85.22-91.45)	84.37 ^{bAB} ±0.37 (81.28-87.06)
Pre Freezing	71.33 ^{c} ±0.39 (68.34-76.38)	81.70 ^{a} ±0.43 (74.87-83.58)	76.88 ^{b} ±0.40 (72.63-79.10)
Post Thaw	56.60 ^{c} ±0.69 (50.74-62.78)	69.71 ^{a} ±0.33 (67.48-73.64)	64.24 ^{b} ±0.52 (61.19-68.65)

(T1=0.5mM Glutathione, T2=1.0mM Glutathione; Means with different superscripts in small letters differs significantly (P<0.05); AB superscript denote the difference between the bulls (unpaired't-test', t=0.19).

3.2 Progressively motile spermatozoa

The mean percentage of progressively motile spermatozoa after dilution in control and treatment groups T1 and T2 was 72.21±0.40, 80.88±0.41 and 76.58±0.45 respectively and it was was significantly (P<0.05) higher in treatment groups T1 and T2 as compared to control group. At pre-freezing stage mean percentage of progressively motile spermatozoa at pre-freezing stage in control and treatment groups T1 and T2 were 64.50±0.55, 74.46±0.53 and 69.92±0.47 respectively and it

was significantly (P<0.05) higher in treatment groups T1 and T2 as compared to control group. At post-thaw the mean percentage of progressively motile spermatozoa was 47.79±0.65, 60.08±0.64 & 55.21±0.6 in control and treatment groups T1 and T2 respectively and it was significantly (P<0.05) higher in treatment groups T1 and T2 as compared to control group. Further, the treatment groups T1 and T2 also differed significantly (P<0.05) and comparison (unpaired 't' test) between the bulls for control and treatment groups did not revealed any significant difference at all stage of semen preservation and at post-thaw (Table: 2).

Table 2: Progressive Motility of Spermatozoa in Cryopreserved Hariana Bull Semen Supplemented with Glutathione (Percent Mean ± SEM, N=24)

Progressive Motility of Spermatozoa			
Stage of Semen Preservation	**Control (0.0mM)**	**Treatment 1 (0.5mM)**	**Treatment 2 (1.0mM)**
After Dilution	72.21 ^{c} ±0.40 (68-75)	80.88 ^{a} ±0.41 (78-85)	76.58 ^{b} ±0.45 (72-80)
Pre Freezing	64.50 ^{c} ±0.55 (60-68)	74.46 ^{a} ±0.53 (68-78)	69.92 ^{b} ±0.47 (65-73)
Post Thaw	47.79 ^{c} ±0.65 (43-52)	60.08 ^{a} ±0.64 (55-65)	55.21 ^{b} ±0.65 (50-62)

(T1=0.5mM Glutathione, T2=1.0mM Glutathione, Means with superscripts in small letters differs significantly; (P<0.05).

4. Discussion

When normal semen has been handled properly and staining is carried out correctly, the percentage of sperm staining alive is highly correlated with individual progressive motility, but the percentage of motility is usually lower than the percentage of live spermatozoa as many of the live sperm may not have motility (Hafez, 1987) [18].

In the present study the initial mean percent live spermatozoa varies between 94.45±0.48 to 95.17±0.37 and do not differs significantly between bulls. Similar study conducted in 2012 (Kumar, 2012) [23] found a range 85.89±1.16 to 90.03±0.83 percent & in 2013 (Sachan, 2013) [40] revealed a range 84.75±2.52 to 90.38±0.91 which is nearly similar with our findings. In Hariana bull the mean live percentage spermatozoa has been reported to vary between 70.45 to 79.2 (Tomar and Kanaujia., 1970, Tomar and Gupta, 1984) [49, 48]. Bhosrekar (1980) [7] reported different proportion of live spermatozoa in different season, which were 67.80, 79.80 and 75.50 percent in winter, summer and monsoon seasons respectively. Rao *et al.,* (1996) [37] reported that semen of Ongole bulls had 84.60 noneosinophilic sperm percentage and was higher than Jersey with 83 percent and Jersey x Ongole with 83.6 percent. Mohan *et al*. (1976) [28] found live spermatozoa of Murrah buffalo bull semen as 82.70% in winter and 83.60% in summer. Mondal (1998) [30] found that the average non-eosinophilic sperm count as 82.07% in Murrah buffalo bulls.

Bhalde *et al.,* (1991) reported non-eosinophilic percent value of 60.76 ± 0.68 and 57.64 ± 0.78 in post-thawed semen after thawing at 35°C for 30 sec and at 5°C for 5 min, respectively. The addition of antioxidant like glutathione might have protective action rendering better livability per cent in respective groups (Uysal *et al*., 2007) [52]. In the present study Glutathione was found to effect significantly on percent live spermatozoa in treatment group 1(0.5mM Glutathione) compare to control and treatment group 2(1.0mM Glutathione) after dilution. The result was subsequently followed in the stage of pre freezing and was more pronounced at post thaw stage. The concentration of 1.0mM Glutathione was found to have a lower viability compare to 0.5mM, however, was having protective action compare to control. Bull effect was observed at dilution stage only and was not seen after this stage. Similar finding has been reported by Singh *et al.* (1997) [47], Gadea *et al*. (2005b) [14], Uysal *et al*. (2007) [52], and Perumal (2008) [35] in EYTG diluter.

Higher initial progressive motility has better freezability (Saxena and Tripathi, 1979) [43]. Kumar, 2012 [23] found a range 73.00±1.11 to 80.00±0.00 percent and Sachan, 2013 [40] found a range between 76.63.±1.45 to 79.38±1.19 percent progressively motile spermatozoa. In the present study the initial progressive motility ranges between 87.83±1.19 to 87.92.±0.35 to percent and do not differs significant between the bulls. Bhosrekar and Nagarcenkar (1971) [8] recorded motility range 20-80% in 514 ejaculates in Murrah buffalo bull. Gopalakrishna and Rao (1978) [16] reported 81.5% initial motility in Murrah buffalo bulls per ejaculate with 4 days semen collection interval. Saxena *et al.* (1978) [44] reported initial motility of 73.17 % in the semen of Murrah Buffalo bulls on the basis of 30 ejaculates. Matharoo and Singh (1980) [26] reported average initial motility of 71.4 % in 52 ejaculates from 4 Murrah buffalo bulls. Shetti *et al.* (1981) [46] collected 30 ejaculates from 6 Murrah buffalo bulls and found 79.45 % progressive sperm motility in freshly diluted semen. Good quality semen should contain 70 to 90 per cent progressively motile sperms. Initial motility of Murrah buffalo bulls was found to be 65 to 86 per cent (Pramanik, 1996) [36].

It has been observed that a spermatozoon in liquid medium is propelled forward by flagellar beating initiated from distal centriole through electron transport. During ultra low freezing the spermatozoa is exposed to cold shock resulting in damage to the sperm membrane and leakage of enzymes. All these adverse factors might be mitigating by incorporation of Glutathione in the semen which act as a membrane stabilizer (Maxwell and Stojanov, 1996) [27]. In the present study addition of Glutathione at a concentration of 0.5mM has resulted in preserving the percentage of progressively motile spermatozoa better compare to 1.0mM Glutathione or control group at any of the stage of cryopreservation and following thawing. Addition of 1.0mM Glutathone has proved better compare to control. The present observation of higher motility in Glutathione added semen is comparable to the findings of Jain and Arora (1988) and Uysal *et al.* (2007) [52]. However slightly lower value compare to our results has also been reported by Zekariya Nur *et al.* (2005) [56] and Munsi *et al.* (2007) [31] in crossbred bulls. Sperm motility was recorded higher with Glutathione at 0.5, 1.0 and 2.0mM while 3mM of glutathione was found non-beneficial (Munsi *et al.,* 2007) [31]. Therefore, 0.5mM of Glutathione was recommended for use in extender for storage of bull semen.

5. Summary and Conclusion

Following evaluation of fresh semen and those which confirmed the standard as stated vide supra, the semen sample of each bull were split into three equal parts and extended with three different combination of dilutors i.e. GEYT (control), GEYT with 0.5mM Glutathione (T1) and GEYT with 1.0mM Glutathione (T2). These samples were then processed for cryopreservation and thawed thereafter. The mean percentage of sperm viability at post-thaw was 56.60±0.69, 69.71±0.33 and 64.24±0.52 percent in control,

tratment 1 and treatment 2 respectively and differes significantly (P<0.05) between treatments and control and higher viablilty of sperm was found in 0.5mM Glutathione. Mean percentage of post thaw progressive motility was 47.79±0.65, 60.08±0.64 and 55.21±0.65 percent control, treatment 1 and treatment 2 respectively with higher mitility at 0.5mM concentration of Glutathione.

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