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Dushyant Yadav
M.V.Sc. DUVASU, Mathura, Uttar Pradesh, India

Vijay Singh
Professor and Head C.V.Sc. and Head CVSc. and A.H. SVPUAT, Meerut, Uttar Pradesh, India

SS Yadav
DUVASU, Mathura, Uttar Pradesh, India

A Patel
Assistant Professor, C.V.Sc. and A.H., SVPUAT, Meerut, Uttar Pradesh, India India

A Kumar
DUVASU, Mathura, Uttar Pradesh, India

Anuj Kumar
DUVASU, Mathura, Uttar Pradesh, India

Sarvjeet Yadav
DUVASU, Mathura, Uttar Pradesh, India

Brijesh Kumar
Scientist, ICAR-IVRI, Izatnagar, Bareilly, Uttar Pradesh, India

RP Pandey
DUVASU, Mathura, Uttar Pradesh, India

Atul Saxena
DUVASU, Mathura, Uttar Pradesh, India

Correspondence
Dushyant Yadav
M.V.Sc. DUVASU, Mathura, Uttar Pradesh, India

Effect of glutathione on viability and acrosomal integrity of bovine spermatozoa during graded cryopreservation

Dushyant Yadav, Vijay Singh, SS Yadav, A Patel, A Kumar, Anuj Kumar, Sarvjeet Yadav, Brijesh Kumar, Brijesh Yadav, RP Pandey and Atul Saxena

Abstract
The present study was aimed to evaluate the effect of Glutathione, an anti-oxidant, on the acrosomal integrity bovine spermatozoa by means of Trypan Blue-Neutral Red- Giemsa Staining at different stages of semen preservation (after dilution, pre-freeze) and post thaw. The semen was primarly examined for standard samples. Samples (N=24) of more than 70% progressively motile spermatozo with concentration of >600 million spermatozoa/ml was used for cryopreservation. The extension of fresh semen was done in a Glycerolated Egg Yolk Tris (GEYT) Extender upto 80 million sperm /ml. The various concentrations of Glutathione were added in diluted semen like 0.0 mM (Control), 0.5 mM (T1) & 1.0 mM (T2). The semen was cryopreserved in French Mini Straw using liquid nitrogen vapour as per standard protocol for patticular time interval (lowering of temperature from 4°C to −10°C @ 5°C/min, −10°C to −100°C @ 40°C/min, −100 to −140°C @ 20°C/min). The acrosomal membrane integrity of spermatozoa was evaluated after staining with Trypan Blue –Neutral red-Giemsa stain at given time and temperature ratio. The viability of spermatozoa with intact acrosome at post-thaw (37^0C for 45 sec) was significantly higher (P<0.05) in the treatment groups (T1) & (T2) and it was 54.43±0.65, 67.58±0.44 & 61.69±0.55 in control, T1 (0.5 mM Glutathione) and T2 (1.0 mM Glutathione) groups respectively. Furtherly, with in two treatments a significant difference was aslo seen (P<0.05) and it was higher in treatment group 1 (T1). Comparison (unpaired't' test) between the bulls for control and treatment groups did not revealed any significant difference.

Keywords: Acrosomal, spermatozoa, cryopreservation

Introduction
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. Cryoinjury to the sperm caused by the cryopreservation procedure leads to poor survival of spermatozoa (Salamon and Maxwell, 1995) [28] and the main site of cryodamage is plasma membrane speciallyi n the principal-piece, midpiece, and head (Holt and North, 1994; Watson, 1995) [16, 39].

The acrosome is a large Golgi/endoplasmic reticulum derived acidic secretary organelle filled with hydrolytic enzymes. The presence of normal acrosome on a spermatozoon is essential for the acrosomal reaction that is being required at the proper time to facilitate fertilization. The change in acrosomal cap is mainly due to sperm aging or cryo-injury. A high correlation between the percentage of intact acrosome and fertility of frozen bovine spermatozoa was observed after 2 and 4 h of post-thaw incubation. Reduction in sperm motility and normal apical ridge (O'Connor *et al.,* 1981) [23], extracellular release of enzymes viz; glutamic oxacloacetic transeminase and acrosin (Graham *et al.,* 1972) [15] and reduced conception rate (Shannon and Vishwanath, 1995) [30] are evidences of damage in bovine semen. It is major part of assessment of functional activity of spermatozoa (Kumar, 2007) [20].

The most commonly used staining method to detect acrosomal changes is Giemsa stain (Watson, 1975). Sharma *et al.,* (1992) [31] observed a positive correlation between the acrosomal integrity with pre-freeze and post- thaw motility. Trypan-blue Giemsa staining describes the live/dead status of the sperm and acrosomal integrity simultaneously in the same semen sample and highly correlated (R2 = 0.62, P = 0.02) with *in vitro* fertility (Tartaglione and Ritta, 2004) [36].

The freeze thawing processes decreased the antioxidant potential of the semen during cryopreservation and increased the lipid peroxidation levels and ROS molecules (Kumar *et al.* 2011; Kadirvel *et al*., 2009) [19]. Oxidative stress generally leads to loss of motility,

swelling and and the blebbing of the acrosomal membrane and disruption or increased permeability of the plasma membrane of spermatozoa (White, 1993) [40]. *In vitro* studies have already demonstrated that hydrogen peroxide could reduce the percentage of sperm with intact acrosomes (Garg *et al.,* 2009) [14]. Higher percentage of acrosomal integrity of swine spermatozoa is associated with higher levels of naturally occurring antioxidant in the semen (Gadea *et al.*, 2004) [13]. Naturally occurring antioxidants in semen protect the acrosomal integrity of the spermatozoa by reducing levels of ROS molecules and lipid peroxidation of cell membrane (Cotran *et al.,* 1989) [9]. Naturally occurring antioxidant added to semen extender improved the sperm population with intact acrosomes in caprine and bovine semen (Sinha *et al.,* 1996; Ansari *et al.,* 2010; 2011a; 2011b) [32, 4, 5, 3].

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 (O'Flaherty *et al.,* 1997) [23]. Controlling oxidation by exogenous antioxidants in the extender, such as catalase, greatly helped maintain sperm quality (Foote, 1967). The use of antioxidant in extender is recommended to reduce the cryodamage to spermatozoa (Sansone *et al.,* 2000 and Andrabi, 2008) [28, 2]. It is known that 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; Andrabi *et al.,* 2008) [6, 2], vitamin E (Ball *et al.,* 2001; Andrabi *et al.,* 2008) [2], catalase (El-Sisy *et al.,* 2008;) [9], superoxide dismutase (El-Sisy *et al.,* 2008) [9], glutathione (Sinha *et al.,* 1996; Foote *et al.,*2002; Munsi *et al.,* 2007; Uysal and Bucak, 2007; Buck and Tekin, 2007; Perumal *et al.,* 2008; Ansari *et al.,* 2010; 2011a) [32, 3, 4, 5, 11, 36, 37, 26, 21], butylated hydroxytoluene (Ball *et al.,* 2001; Ansari *et al.,* 2011c, Patel *et al.,* 2016) [6, 4, 5, 25, 3] etc. 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 and exists in two forms: the reduced form (GSH) and the oxidized form (GSSG). The sulphydryl group (SH) of glutathione confers its protective action against oxidative damage. The protective action of glutathione against ROS is facilitated by the interactions with its associated enzymes, such as glutathione peroxidase and glutathione reductase (Storey *et al.,* 1997) [34]. 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) [10, 26].

Material and Method

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. Routien examination of artificially collected was done for standard ejaculates (N=24). 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 (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 with help of biological freezer (IMV, Technologies France), in 7 minutes and 5 second (lowering of temperature from 4°C to −10°C @ 5°C/min, −10°C to −100°C @ 40°C/min, −100 to −140°C @ 20°C/min). Post-thaw evaluation was done after the thawing of semen straw at 37^0C for 45 sec.

Trypan blue-Neutral red-GIEMSA staining for Acrosomal Integrity of Spermatozoa: Staining was carried out as described by Kovacs & Foote (1992) [17] with little modification.

Fixation and staining of spermatozoa: Diluted/Frozen thawed semen was 10 times diluted by adding 1 part of semen to 9 parts of 0.9% NaCl. One drop of 0.27 % trypan blue (made from two parts of a buffered 0.4% solution (Sigma T8154) and one part of 0.9% NaCl) and one drop of diluted semen were mixed on a glass slide and spread over the entire surface by using another slide. Slides were air-dried in vertical position then put into a fixative (mixture of 86ml 1N HCL and 14 ml of 37% formaldehyde solution with the addition of 0.2g Neutral Red) in a coupling jar for two minutes, and then rinsed with tap and distilled water. Slides were put into coupling jars containing the Giemsa staining solution and left overnight (16 to 20 hours) at room temperature. The Giemsa Staining solution was freshly prepared by adding 7.5% (v/v) of Giemsa stock solution to 100 ml distilled water. [500 ml Giemsa stock solution (Sigma GS-500) contains; Giemsa stain-3.8 gm, Absolute alcohol (GR grade)-375 ml and Glycerol (AR grade)-125 ml]. Slides were rinsed again in tap and distilled water for 2 minutes each, air-dried in vertical position. Microscopic evaluation of sperm cells was performed at 100x oil immersion magnification.

Different Types of Acrosomal Inegrity: Based on staining characteristics of sperm cells were differentiated into four categories: AIL (Acrosome Intact Live-Fig: 1, a), AID (Acrosome Intact Dead- Fig: 1, b), ALL (Acrosome Lost Live Fig: 1, c), ALD (Acrosome Lost Dead Fig: 1, c).

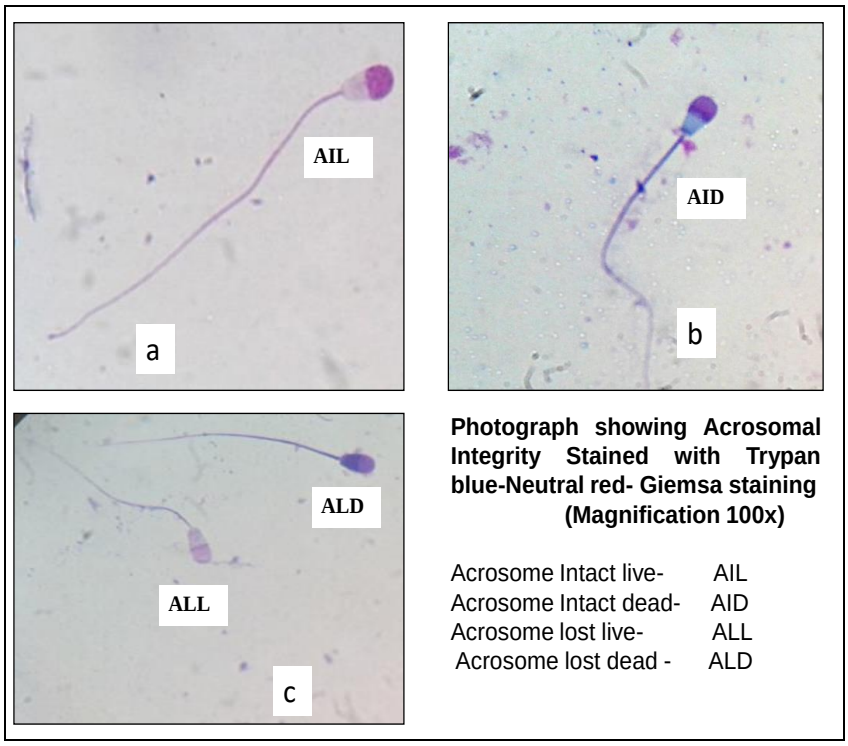

Fig 1: Different status of Acrosomal Integrity and Sperm Viability.

Results

Live spermatozoa with intact acrosome (AIL)

Perusal of Table 1 revealed that following dilution of semen the mean percentage of live spermatozoa with intact acrosome was significantly higher (P<0.05) and it was 78.71±0.32, 86.73±0.50 and 82.37±0.39 in the control and T1 and T2 groups resectively. The mean percentage of live spermatozoa with intact acrosome at pre-freezing stage in control and treatment groups T1 and T2 was 68.87±0.52, 79.58±0.52 and 74.31±0.48 respectively and it was significantly higher in treatment groups (P<0.05). A significantly higher live sperms with intact acrosome was found in the treatment groups after post-thaw evaluation and it was 54.43±0.65, 67.58±0.44 & 61.69±0.55 in control, T1 and T2 groups respectively (P<0.05). Furtherly, with in two treatments a significant difference was aslo seen (P<0.05) and it was higher in T1 group.

Dead spermatozoa with intact acrosome (AID)

After dilution of semen the mean percentage of dead spermatozoa with intact acrosome in control and treatment groups T1 and T2 was 20.64±0.36, 12.04±0.42 and 16.29±0.41 respectively and it was significantly(P<0.05) lower in the treatment groups. Further, the treatment groups T1 and T2 also differed significantly (P<0.05). At pre-freezing stage the mean percentage of dead spermatozoa with intact acrosome in control and treatment groups T1 and T2 were 29.32±0.48, 19.08±0.48 and 23.91±0.49 and it was also significantly (P<0.05) lower in treatment groups along with significant differnce in the two treatments. The post thaw evaluation of mean percentage of dead spermatozoa with intact acrosome in control and treatment groups T1 and T2 was 43.63±0.67, 30.69±0.45 & 36.36±0.53 and significantly (P<0.05) lower in the treatmet groups (Table 1).

Tables 1: Viability and acrosomal integrity of spermatozoa in Hariana Bull semen with Glutathione supplementation during cryopreservation and post-thaw (Percent Mean ± SEM, N=24)

Live Spermatozoa with Intact Acrosome (AIL)			
	Control	**Treatment 1**	**Treatment 2**
After Dilution	78.17 ^{c} ±0.32 (74.25-81.81)	86.73 ^{a} ±0.50 (80.61-89.94)	82.37 ^{b}±0.39 (77.22-85.50)
Pre Freezing	68.87 ^{c} ±0.52 (63.33-74.01)	79.58^{a}±0.52 (72.19-82.75)	74.31^{b}±0.48 (69.71-77.56)
Post Thaw	54.43^{c}±0.65 (50.24-61.76)	67.58^{a}±0.44 (61.40-72.36)	61.69^{b}±0.55 (58.14-68.00)
Dead spermatozoa with Intact Acrosome (AID)			
	Control	**Treatment 1**	**Treatment 2**
After Dilution	20.64 ^{a} ±0.36 (16.66-24.75)	12.04 ^{c} ±0.42 (09.05-16.33)	16.29 ^{b} ±0.41 (12.74-21.28)
Pre Freezing	29.32 ^{a} ±0.48 (23.52-34.31)	19.08 ^{c} ±0.48 (15.58-25.85)	23.91 ^{b} ±0.49 (20.48-28.21)
Post Thaw	43.63 ^{a} ±0.67 (37.73-48.76)	30.69 ^{c} ±0.45 (26.13-35.96)	36.36 ^{b} ±0.53 (31.37-39.90)
Live spermatozoa with Lost Acrosome (ALL)			
	Control	**Treatment 1**	**Treatment 2**
After Dilution	0.43 ^{a} ±0.08 (0.00-0.99)	0.37 ^{b} ±0.09 (0.00-1.01)	0.70 ^{a} ±0.09 (0.00-1.69)
Pre Freezing	0.93 ^{a} ±0.06 (0.00-1.45)	0.62 ^{b} ±0.11 (0.00-1.98)	0.93 ^{a} ±0.04 (0.49-1.31)
Post Thaw	0.93 ^{a} ±0.06 (0.00-1.47)	0.75 ^{a} ±0.08 (0.00-1.31)	0.80 ^{a} ±0.10 (0.00-1.76)
Dead spermatozoa with Lost Acrosome (ALD)			
	Control	**Treatment 1**	**Treatment 2**
After Dilution	0.67 ^{a} ±0.09 (0.00-1.51)	0.52 ^{a} ±0.08 (0.00-1.00)	0.63 ^{a} ±0.09 (0.00-1.48)
Pre Freezing	0.85 ^{a} ±0.09 (0.00-1.48)	0.68 ^{a} ±0.08 (0.00-1.51)	0.86 ^{a} ±0.10 (0.00-1.74)
Post Thaw	1.11 ^{a} ±0.09 (0.49-1.88)	0.77 ^{a} ±0.11 (0.00-1.94)	1.17 ^{a} ±0.11 (0.49-2.64)

Live spermatozoa with lost acrosome (ALL)

Table 1 revealed that following dilution the mean percentage of live spermatozoa with lost acrosome was significantly (P<0.05) lower in treatment group T1 as compared to treatment group T2. However, control group was neither significantly lower from treatment group T1 nor from treatment group T2 and it was 0.43±0.08, 0.37±0.09 and 0.70±0.09 in control and treatment groups T1 and T2 respectively. At pre-freezing stage the mean percentage was significantly (P<0.05) lower in treatment groupT1 as compared to control treatment group T2 and it was 0.93±0.06, 0.62±0.11 and 0.93±0.04 respectively. At post-thaw evaluation the mean percentage revealed no any significant difference between treatment and control.

Dead spermatozoa with lost acrosome (ALD)

The mean percentage of dead spermatozoa with lost acrosome did not differ significantly between the treatment and control groups at any stage of semen preservation and at post-thaw also.

Comparison (unpaired‘t’ test) between the two bulls for control and treatment groups did not revealed any significant difference at any stage of semen preservation and also at post-thaw in terms of acrosomal integrity of spermatozoa.

Discussion

The maintenance of acrosomal integrity is important for sperm movement and fertilization while preserving the enzyme integrity of sperm head. During the sperm penetration at the time of fertilization the essential enzymes like acrosin, proacrosin and hyaluronidase play an important role for dissolution of membrane layer of an ovum. Hence, any alteration in the structural integrity may lead to loss of these enzymes which ultimately reduces the sperm capability of penetration of oocytes layers leading to fertilization failure. The processes of cryopreservation including cooling, freezing and thawing create oxidative stress on the spermatozoa membrane (Aitken *et al.*, 1998, White, 1993; Chatterjee *et al*., 2001) [1, 40, 7]. Oxidative stress generally leads to loss of motility, swelling and the blebbing of the acrosomal membrane and disruption or increased permeability of the plasma membrane of spermatozoa (White, 1993) [40]. As the spermatozoa are subject to lower temperature these changes may aggravate leading to disruption of acrosomal membrane of sperms through the process of cryopreservation and thawing leading to lower count of sperms with intact acrosomes.

Bull semen diluent with glutathione incorporation has been reported to improve post thaw motility and reduces the acrosomal damage. It has also been reported to reduce enzyme leakage and consistently improved fertility. The mode of action might be due to limited the production of lipid peroxides (Kumar, 2007) [20]. Boccia *et al*. (2007) [20] also observed an increase in percentage of intact acrosome after the addition of antioxidant (Glutathione) as compare to control group. 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 3.0mM (Munsi *et al.*, 2007) [21]. In this study sperm motility, normal acrosome, mid piece, tail and head abnormalities were assessed before and after freezing and on consecutive days of storage up to the 5th day. Sperm motility was recorded higher with glutathione at 0.5, 1.0 and 2.0 mM while 3mM of glutathione 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. Addition of 1.0 mM of BHT results in a significant (p<0.05) improvement in sperm viability and acrosomal integrity during cryopreservation (Patel *et al.*, 2016) [25]. Addition of Iodixanol (an oxidant) reduces capacitation like changes during cryopreservation of spermatozoa in Bhadawari buffalo bull spermatozoa (Yadav *et al.*, 2016) [24]. In the present study the live spermatozoa with intact acrosome was significantly higher (P<0.05) in treatment group T1 as compare to the control and treatment group T2.

Summary and Conclusion

Addition of 0.05 mM (T1) Glutathione in the extender during cryopreservation of Hariana bull semen was found signinificantly more benificial in terms of acrosomal integrity and sperm viability (Live spermatozoa with intact acrosome) 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 prevents the cryo-damage in therm of acrosomal integrity of bovine sprematozoa and improoves the sperm viability.

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