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Effect of glutathione on leakage of various enzymes from buck semen during freezing

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ABSRACT

Semen samples from 12 bucks were extended in Tris dilutor with 2 and 5 mM of glutathione. The activities of acrosome, hyaluronidase, AKP, AST, ALT and LDH were assayed in equilibrated and frozen-thawed semen samples. Significantly ($P < 0.01$) higher intracellular activity of acrosin was recorded in semen samples extended with 5 mM of glutathione. Effect of glutathione on acrosin activity was significant at both the pre and post-freeze stage. Addition of 5 mM glutathione also decreased significantly the release of hyaluronidase, AKP, AST, ALT and LDH enzymes from the sperm cell in the medium.

Key words : Goat, glutathione, acrosin, hyaluronidase, frozen semen

Freezing and thawing inflict considerable damage to sperm plasma lemma with concomitant leakage of various enzymes as well as other materials (Salisbury, *et al.*, 1978). Acrosin and hyaluronidase the two important enzymes of acrosome system play a pivotal role during fertilization and their leaking due to cellular damage during freezing/thawing is detrimental for the fertilization process. Besides this, alkaline phosphatase (AKP), transaminases (aspartate aminotransferase and alanine aminotransferase) and lactic dehydrogenase (LDH) are also essential for metabolic processes which provide energy for survival, motility and fertility of spermatozoa. It is well known that during storage, freezing and thawing of spermatozoa, sperm phospholipids undergo peroxidation which leads to formation of toxic fatty acids peroxides. These peroxides further lead to the structural damage to the sperm cell accompanied by lower motility and metabolism and enzyme leakage (Jone and Mann, 1977 and Mann *et al.*, 1980). Glutathione, as a substrate of glutathione peroxidase present in the sperm head, slows down the peroxidation process and hence stabilises the cell membrane (Christopherson, 1968). In the past therefore, attempts have been made to improve the motility of spermatozoa as well as to check enzyme release by incorporating glutathione as an additive in chilled and frozen bull semen (Shanon *et al.*, Slaweta and Laskowska, 1987). Reports on the effects of glutathione on goat sperm are lacking. The present study was therefore undertaken to assess the effect of different levels of glutathione on post thaw enzyme release from frozen goat sperms of different breeds.

MATERIALS AND METHODS

Seventy two ejaculates were obtained from 12 healthy bucks (three Black Bengal, three Beetal and six their crosses) by means of an artificial vagina. Immediately after routine evaluation, the semen, samples were diluted in Tris egg yolk extender containing Tris (hydroxy methyl aminomethane, 12.10 gm), fructose (5.0 gm), citric acid (6.8 gm), glycerol (32.0 ml) and distilled water (368 ml). Extended samples were divided into three equal fractions, one of which served as control. In the other two fractions, reduced glutathione (GSH, Sigma Chemical Co., St. Louis, Mo, USA) was added at concentration of 2 and 5 mM. All the samples were packaged in Minitubes (Vol. 0.28 ml containing about 3 million sperms), equilibrated at 5±1°C for 5 hour and frozen by an instant freezing technique (Bhandari *et al.*,. 1982).

Activities of acrosine, hyaluronidase, AKP, aspartate transaminase (AST), alanine transaminase (ALT) and LDH were assayed in equilibrated and frozen-thawed semen samples. Acrosin activity (intact with the sperm cells) was estimated as intracellular total proacrosin (Amann *et al.*, 1986) which was released by incubating the sperm pack (109 spermatozoa) overnight at 4°C in the benzamidine medium (pH 2.8) containing 10.0% glycerol (Polakoski *et al.*, 1977). Subsequent to incubation, the samples were

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centrifuged (3000 rpm for 10 minutes) and the supernatant was utilized for the measurement of acrosin activity which was determined following hydrolysis of Benzyl-arginine-o-nitronilide (BAPNA, extinction coefficient 8800) and was expressed as μM BAPNA hydrolysed/min/109 spermatozoa., Extracellular hyaluronidase activity was estimated by the method of Linker (1956) and expressed as μM-N-acetyl glycosamine liberated/min/109 spermatozoa. The extracellular activity of AST and ALT was determined by the Reitman and Frankel (1957) method, AKP by Kind and King (1954) method and LDH by Cabaud *et al.* (1958) method on the suspension medium using the diagnostic kits (Span Diagnostic Private Limited, Surat, India).

Analysis of variance was performed to study the effect of glutathione on different enzyme activities for pre and post freeze storage.

RESULTS AND DISCUSSION

The mean activity of enzymes in pre and post-freeze semen samples are presented in table 1. The intracellular acrosin activity was significantly higher ($P < 0.01$) in the extender containing 5 mg of glutathione than in Tris or Tris + 2 mg glutathione, both at pre and post freeze stage. Freezing procedure has been reported to alter cell permeability and damage acrosome, causing release of enzymes (McRorie and Williams, 1974 and Singh *et al.*, 1995). During the present study acrosin activity was found to decrease from pre-freeze stage to after freeze stage which was lowest in 5 mM glutathione added semen. The extracellular activity of hyaluronidase has been recorded to increase from the pre-freeze stage to post-freeze stage and varied among extenders and was significantly lowest ($P < 0.01$) in the 5 mM added glutathione semen. Hyaluronidase is an another important enzyme of the acrosome system and its release into the medium from acrosome is an early and sensitive indicator of acrosomal damage (Foulkes and Watson, 1975). Likewise in the suspension medium of sperm preserved, the activity of AKP, AST, ALT and LDH increased markedly in all the extenders from the pre-freeze to post-freeze stage. However, activities of all above enzymes were significantly lower ($P < 0.05$) in the presence of glutathione (5 mM) at both the stages of preservation (Table 1). An increase in extracellular activity of Hyaluronidase, AKP, AST, ALT and LDH from the pre-freeze to the post-freeze stage has been reported by many workers in bulls (Buruiana *et al.*, 1980), rams & goats (Rao *et al.*, 1984 and Singh *et al.*, 1995).

In our investigation the intracellular level of acrosin was highest and the release of hyaluronidase, AKP, AST, ALT and LDH enzymes in the medium were lower with 5 mM glutathione both at pre and post-freeze stages which agrees with the report of Slaweta and Laskowska (1987). Mammalian spermatozoa are highly sensitive to lipid peroxidation which occurs as a result of oxidation of membrane lipids by partially reduced oxygen molecules. Lipid peroxidation is also affected by H_2O_2 produced as a result of oxidative deamination of aminoacids in semen. Toxic oxygen metabolites are emerging as a final common pathway of cell injury in varying processes such as chemical and physical injury as well as cellular ageing (Cotran *et al.*, 1989). Intracellular oxygen, in conjunction with a number of oxidative enzymes produces partially reduced

Table 1. Mean acrosin, hyaluronidase, AKP, AST, ALT and LDH level in pre and post-freeze semen samples preserved with and without glutathione

Enzyme	Extender	Tris	Tris + 2 mm glutathione	Tris + 5 mm glutathione
Acrosin	Pre freeze	1.784±0.042^c	2.249±0.037^b	2.340±0.029^a
	Post freeze	1.488±0.043^c	1.556±0.039^b	1.715±0.034^a
Hayluronidase	Pe freeze	274.72±0.760^a	263.677±2.483^b	257.97±2.786^b
	Post freeze	425.62±4.984^a	406.771±5.299^b	500.648±5.332^b
AKP	Pre freeze	8.286±0.578^a	5.081±0.328^b	4.830±0.301^b
	Post freeze	34.492±1.235^a	24.920±0.654^b	19.253±0.882^c
AST	Pre freeze	93.095±0.863^a	85.711±0.718^b	77.746±1.150^c
	Post freeze	286.079±3.417	262.658±2.683^b	233.650±3.360^c
ALT	Pre freeze	64.174±0.927^a	59.277±0.637^b	54.936±0.826^c
	Post freeze	211.553±2.717^a	203.214±1.97^b	191.952±2.782^c
LDH	Pre freeze	292.269±3.655^a	277.698±2.459^b	260.460±3.068^c
	Post freeze	1639.861±11.431^a	1598.19±10.961^b	1503.513±7.812^c

Values bearing different superscript in a row differ significantly ($P < 0.01$)

toxic oxygen molecules such as superxode, H_2O_2 and hydroxyl radicals. These free radicals must be destroyed by antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase if damage to the cell membrane by lipid peroxidation is to be prevented (Cotran *et al.*, 1989). Lipid peroxidation is further initiated by hydroxyl radicals which in turn react with unsaturated fatty acids and generate organic free radicals which inturn react quickly with oxygen to form lipid peroxides. These lipid peroxides then themselves act as free radicals initiating an autolytic chain reaction resulting in further damage to the cell membrane (Cotran *et al.*, 1989). It is important to note that lipid peroxidation and subsequent membrane damage is at its peak during the semen thawing process. Although the exact mechanism is not clear, there is now evidence that free radical toxic oxygen metabolites are produced at low levels in cells with a restricted supply of oxygen but there is an increase in free radical production on restoration of oxygen supply to the cell (Cotran *et al.*, 1989). Hence it is speculated that the sudden increase in oxygen utilization by spermatozoan during thawing, following the dormant metabolic stage, might be responsible for increased production of free radicals, leading to increased lipid peroxidation and thus spermatozoal damage.

The results of our present study on release of enzymes from sperm cells confirms that the process of freezing damages the cell membrane and leads to leakage of these enzymes into the medium. During the present study the extent of leakage of enzymes was low in semen extended with 5mM glutathione. Since glutathione plays an important role in scavenging reactive oxygen intermediates and other free radicals with the help of glutathione reductase enzyme (Meister and Anderson, 1983), it is quite reasonable to believe that glutathione might be protecting spermatozoan from membrane damage by inhibiting the lipid peroxidation process.

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