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Research Article

Studies on cryopreservation of cauda epididymal spermatozoa in buffaloes

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ABSTRACT

The effect of cryopreservation on the viability, motility, acrosomal integrity, cervical mucus penetration ability and response to hypo-osmotic solution was studied on samples of cauda epididymal semen collected from 12 mature buffalo bulls slaughtered at the local slaughter house. Though a significant reduction ($P<0.05$) in all the parameters was observed after cryopreservation it was concluded that successful cryopreservation of the cauda epididymal semen can be done. The viability, motility, acrosomal integrity, cervical mucus penetration ability and response to hypo-osmotic solution were reduced from 83.92±0.80%, 72.75±0.91%, 80.42±0.82%, 21.17±0.75 mm and 72.00±0.93% to 68.67±0.94%, 51.58±1.18%, 66.83±1.05%, 17.67±0.84 mm and 59.67±0.85%, respectively.

Key words- Buffalo, cauda epididymal spermatozoa, cryopreservation.

Cryopreservation of epididymal spermatozoa is a seldom-practiced technique and limited information is available on the successful cryopreservation of buffalo cauda epididymal spermatozoa. Cryopreservation of cauda epididymal spermatozoa does not offer much advantage over cryopreservation of ejaculates under routine circumstances and moreover the method is cumbersome with respect to its collection from a live animal. However, it holds promise in the recovery of valuable germplasm from a genetically superior bull in the case of its accidental death. The canalization of the cauda epididymis of live animal is possible for collection of appreciable quantities of epididymal semen from elite bulls with acquired defects unable to ejaculate. The technique is important for experimental models where spermatozoa free from seminal plasma is needed in order to assess modulation of sperm properties by seminal plasma constituents and their effects on cryopreservation.

The bubaline cauda epididymal semen has a volume in the range of 0.5-1.5 ml (Gupta, 1992). Bennet and Rowson (1963) reported that bovine cauda epididymal semen contained 7.6 billion sperm per ml. Bovine spermatozoa are stored in cauda epididymis in a quiescent state (Mann and Lutwak-Mann, 1981), probably to conserve energy (Zaneveld and Chatterton, 1982). The cauda epididymal spermatozoa become fully motile on dilution with balanced solutions including seminal plasma but not in the cauda epididymal fluid (Carr and Acott, 1984). The initial motility and livability of buffalo cauda epididymal semen has been reported to be 50–80% and 60 – 90% respectively (Gupta, 1992). Lambrechts *et al.* (1999), in two experiments with African buffalo epididymal spermatozoa found the intact acrosome to be 89.3 ± 2.3 and 93.5 ± 2.2%.

TRIS diluent with 5% as well as 20% egg yolk has been used for dilution of cauda epididymal semen (Gupta, 1992; Graham, 1994) and glycerol at a concentration of 7% has been used cryopreservation of epididymal semen (Graham, 1994). A total equilibration period of 3 hr with the final temperature of 5°C has been found to be effective in cryopreservation of cauda epididymal spermatozoa (Gupta, 1992). TALP media was used for the cryopreservation of African buffalo cauda epididymal spermatozoa (Lambrechts *et al.*, 1999).

MATERIALS AND METHODS

The study was conducted under field conditions and methods employed were chosen to meet the specific needs.

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Collection, processing and freezing of cauda epididymal semen

Epididymal semen was collected from 24 epididymis of 12 adult buffalo bulls slaughtered at the National Buffalo Slaughter House, Bareilly (UP). The testes along with epididymis from each animal were removed immediately after slaughter and transported to the laboratory in normal saline. At the laboratory the testes were cleaned under tap water and sterilized with 70% alcohol. The epididymides were dissected free of testes and then placed individually in large Petri dishes. The epididymides were then carefully separated. Epididymal ducts were then exposed by trimming the superficial tissue and were punctured at many points by a 16-gauge hypodermic needle. Cauda region of epididymis was squeezed and the oozing fluid rich in spermatozoa was collected in Tris dilutor (Tris hydroxy methyl amino methane, 3.028 g; Citric acid monohydrate, 1.675 g; Fructose, 1.25 g; Penicillin G sodium, 500 – 1000 IU/ml; Streptomycin sulphate 500 – 1000 µg/ml; Double glass distilled water up to 100 ml). The spermatozoa from the same pair of testes were combined and the suspended semen samples were washed twice. Initial washing was done for 10 minutes at 500 rpm and the pellet obtained was suspended in Tris dilutor. The second washing was done for 10 minutes at 1000 rpm. The epididymal semen pellets were further diluted with Tris-egg yolk-Glycerol dilutor (Tris (hydroxy methyl) amino methane, 3.028 g; Citric acid monohydrate, 1.675 g; Fructose, 1.25 g; Penicillin G sodium, 500 – 1000 IU/ml; Streptomycin sulphate 500 – 1000 µg/ml; Double glass distilled water up to 100ml; Glycerol, 7% and Egg yolk, 10%) such that the concentration of spermatozoa was 30,000 /ml after dilution.

The epididymal semen was then packed in straws (0.5 ml) and subjected to a combined cooling cum equilibration period of 3 hr at 5°C. The straws were kept in liquid nitrogen vapour until temperature of epididymal semen reached -110°C to -114°C. The straws were held at this temperature for 10 minutes and later on plunged into liquid nitrogen (-196°C) for storage.

Epididymal semen evaluation

Epididymal semen samples were taken immediately after the equilibration period for prefreeze evaluation. Frozen straws were thawed after 24 hr of freezing at 37°C for 30 seconds for post-thaw evaluation.

Evaluation of epididymal semen samples for progressive motility, liveability, acrosomal integrity and the in vitro fertility tests i.e. bovine cervical mucus penetration test (BCMPT) and (HOST) were conducted at prefreeze and post-thaw stages. Sperm motility was determined under a phase contrast microscope (40×) using a biotherm stage. Livability of spermatozoa was assessed using Eosin-Nigrosin staining technique (Campbell *et al.*, 1953) and the acrosomal integrity assessed by staining with Giemsa (Watson, 1975). The cervical mucus penetration test was performed as per the method described by Kremer (1965) and Matousek *et al.* (1989). The incubation of test system was carried out at 37°C for a period of 1 hr. The HOST was performed as per the method described by Jeyendran *et al.* (1984) with some modification. The osmolarity of hypo-osmotic solution was adjusted with distilled water at 100 mOsm/l.

The data obtained were subjected to analysis as per the standard procedure of Snedecor and Cochran (1989).

RESULTS AND DISCUSSION

The seminal characteristics for the cauda epididymal spermatozoa suspended in Tris buffer before being subjected to cooling and results observed in the study at the prefreeze and post thaw stages in table 1. A significant ($P<0.05$) decrease was observed in the progressive motility, viability and acrosomal integrity of the frozen-thawed cauda epididymal spermatozoa when compared to the pre-freeze values (fig 1). These results were in agreement with the findings of Gupta (1992) who found a drastic reduction in the viability, motility and acrosomal integrity of the cauda epididymal spermatozoa upon cryopreservation. Mammalian spermatozoa are very sensitive to cooling from body temperature to near freezing, which is regarded as cold shock (Parks, 1997). Ultrastructurally, cold shock is manifested clearly by a disruption of the acrosomal membranes. Cold shock etiology involves damage to the

cellular membrane and alteration in the metabolic function, probably caused by alterations in the arrangement of membrane constituents (Parks, 1997). A thermotrophic phase transition occurs in the membrane phospholipids resulting in more rigid membrane structure. The transition leads to rearrangement of membrane components leading to formation of microdomains of non-bilayer forming lipids. These alterations predispose the apposing membranes to fuse and affect protein activity on thawing. It therefore leads to altered permeability to water and solutes (Mediros *et al.*, 2002). As the temperature reaches about -10°C the risk of intracellular water freezing and consequent disruption of cellular structures from the mechanical stress of ice crystal formation makes it a critical range (Gao *et al.*, 1997; Karow, 1997). Severe dehydration of the cells leads to solution- effect injury, which may lead to irreversible membrane collapse (Gao *et al.*, 1997). All this could lead to an increased cryodamage, which is mirrored by decreased sperm motility, viability and acrosomal integrity.

A substantial reduction ($P<0.05$) in the HOST response was seen in the cryopreserved semen in comparison to the pre freeze semen (fig 1). This can be attributed to the mechanical damages, which increase the membrane permeability and subsequent loss of plasma integrity associated with osmotic and chemical damages (Carreras *et al.*, 1992).

Cryopreservation and thawing resulted in a significant decrease ($P<0.05$) in the penetration of the cauda epididymal spermatozoa ability (fig 1). The values obtained reflect the positive correlation of cervical mucus penetration with motility and livability of semen sample. The samples in which there was a drastic reduction of motility and livability there was a reduction in the mucus penetration distance as well.

Table 1 Effect of cryopreservation on the epididymal semen characteristics (n = 12)

Parameters	Stage of Processing		
	Initial	Pre freeze	Post-thaw*
Progressive motility (%)	78.83±0.64	72.75±0.91	51.58±1.18
Livability (%)	87.67±0.73	83.92±0.80	68.67±0.94
Acrosomal integrity (%)	83.91±0.80	80.42±0.82	66.83±1.05
In vitro fertility tests			
BCMPT (mm)		21.17±0.75	17.67±0.84
HOST (%)		72.00±0.93	59.67±0.85

* Values of all the parameters differ significantly at the post-thaw stage from the pre-freeze stage

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