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Cryopreservation of Sahiwal Bull Epididymal Spermatozoa

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Abstract.- The objective of this study was to evaluate tris-citric egg yolk cryodiluter for cryopreservation of Sahiwal bull epididymal spermatozoa. Sahiwal bull epididymal spermatozoa were recovered from eight slaughtered bulls. Semen retrieved from cauda of each testis pair was pooled and diluted in tris-citric egg yolk extender having 30 × 10^6 spermatozoa ml^{-1}. After dilution semen was filled in 0.5 ml straws, cooled to 5°C within two hours and equilibrated for four hours at 5°C. The straws were held at liquid nitrogen vapours for 10 minutes and then plunged into liquid nitrogen for storage. Sperm motility, plasma membrane and acrosomal integrity of each semen sample were assessed post thaw. Sperm motility of Sahiwal epididymal spermatozoa was 50.6±1.5, 33.8±0.9 and 18.1±1.3 percent at 0, 2 and 4 hour post thaw, respectively. Percentage of intact plasma membrane was observed 70.5±1.4, 52.9±1.0 and 40.0±1.6 at 0, 2 and 4 hour post thaw, respectively. The percentage of spermatozoa with normal acrosomes was 79.1±0.4, 75.1±0.2 and 71.5±0.2 at 0, 2 and 4 hour post thaw, respectively. In conclusion, tris-citric egg yolk cryodiluter may be used for the cryopreservation of Sahiwal bull epididymal spermatozoa.

Keywords: Sahiwal bull, epididymal spermatozoa, cryopreservation, cryodiluter

INTRODUCTION

Sahiwal is one of the indigenous breeds of South Asia which is considerably declining in number (Dahlin *et al*., 1998) because of indiscriminate crossbreeding with exotic breeds. However, it has also been recognized that crossbred animals have poor adaptability to the local environment (Rehman *et al*., 2006; Garcia *et al*., 2003; Joshi *et al*., 2001).

Drastic decline in the purebred Sahiwal population needs special considerations to conserve the valuable germplasm of this indigenous breed (Dahlin *et al*., 1998). To achieve this end, the Government of Punjab, Pakistan, established an independent organization "Research Centre for Conservation of Sahiwal Cattle" with the charter to conserve Sahiwal pure breed. The role of assisted reproductive biotechnology is of significance in the conservation of wild and livestock species (Andrabi *et al*., 2007; Martins *et al*., 2007). Cryopreservation of epididymal spermatozoa from accidentally dead animal and use of this germplasm by artificial insemination are the good options for the propagation of this breed of cattle. Successful cryopreservation of the epididymal spermatozoa of Sahiwal bull needs suitable cryodiluent capable of protecting sperm from cryo-damage.

Routinely, tris-citric egg yolk cryodiluter containing glycerol and egg yolk as cryoprotectant have been widely used for the cryopreservation of bovine ejaculated semen. However this extender has not been tested for the cryopreservation of Sahiwal bull epididymal spermatozoa. The objective of this study was to evaluate the suitability of tris-citric egg yolk cryodiluter for motility, plasma membrane and acrosomal integrity of Sahiwal bull epididymal spermatozoa after cryopreservation.

MATERIALS AND METHODS

Preparation of cryodiluent

Tris-citric egg yolk based cryodiluent was used as an experimental extender. Tris-citric egg yolk extender consisted of 1.56 g citric acid (Merck, Germany), 3.0 g tris–(hydroxymethyl)-aminomethane (Sigma, USA), 74 ml distilled water, fructose 0.2% (wt/vol; Riedel-DeHaen, Switzerland), glycerol (7%; vol/vol; Merck,

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Germany) and egg yolk 20% (vol/vol). The pH of buffer was 7.0 and the osmotic pressure was 320 mOsmol Kg^{-1}. Antibiotics; streptomycin sulphate @ 1mg/ml, procaine penicillin @ 300 IU/ml, benzyl penicillin @ 100 IU/ml available as Sinbiotic® (China) were added.

Epididymal sperm collection and initial evaluation

Eight pairs of testes from mature Sahiwal bulls of uniform age slaughtered at local slaughter house were transported to the Laboratory in Styrofoam box (Coleman®, USA) at 25°C within four hours for the collection of epididymal sperm. Each pair was used for a single set of experiment. The spermatozoa were obtained by slicing and squeezing the epididymis, in 15 ml plastic tube (Martins *et al*., 2007). Visual motility was assessed microscopically (400X; Olympus BX20). Sperm concentration was assessed by Neubauer haemocytometer (Akhter *et al*., 2008).

Epididymal semen processing

The spermatozoa collected from each pair of testes were pooled and diluted with extender in a single step (30 X 10^{-6} motile spermatozoa/ml). Diluted semen was packed in straws (0.5 ml, IMV, France), subjected to cooling to 4°C and equilibrated for four hours. The straws were kept above liquid nitrogen vapours for 10 minutes and then plunged into liquid nitrogen (-196°C) for storage. After 24 hours, semen straws were thawed at 37°C for one minute in water bath and then incubated for 4 hours at 37°C for assessment of post-thaw semen quality.

Post-thaw sperm function assays

Visual motility

Visual motility of Sahiwal bull epididymal spermatozoa was recorded at 0, 2 and 4 h post-thaw. A 5 µl drop of thawed semen was placed on a warmed (37°C) glass slide and cover-slipped. Visual motility was assessed under phase contrast microscope (400X; Olympus BX20; 37°C) attached with a closed circuit television (Akhter *et al*., 2008).

Plasma membrane integrity

Plasma membrane integrity of Sahiwal bull epididymal spermatozoa was assessed by supravital stain Trypan blue (0.4%, in distilled water) at 0, 2 and 4 h post thaw (Brito *et al*., 2003). The assay was performed by mixing 5µl semen sample with equal volume of Trypan blue solution on slide and air dried for 10 minutes. A total of 100 sperm was examined under phase contrast microscope (400X; Olympus BX40, Japan). Sperm stained blue were categorized having damaged plasma membrane while clear sperm were considered with intact plasma membrane.

Acrosomal integrity

Acrosomal integrity of Sahiwal bull epididymal spermatozoa was determined at 0, 2 and 4 h post-thaw. To assess the acrosomal intactness, semen (100 µl) was fixed in 1% formal citrate (500 µl; 2.9 g tri-sodium citrate dihydrate; Merck, Germany and 1 ml, 37% solution of formaldehyde; Merck, Germany dissolved in 99 ml of distilled water). Intactness of acrosome was characterized by normal apical ridge. One hundred spermatozoa were studied using phase contrast microscope (1000X; Olympus BX40, Japan) under oil immersion (Akhter *et al*., 2008).

RESULTS

The data on post thaw sperm motility, plasma membrane integrity and normal apical ridge of Sahiwal bull epididymal spermatozoa at 0, 2 and 4 hour at 37°C are given in Table I. Percentage of Sperm motility of Sahiwal bull epididymal spermatozoa was 50.6±1.5, 33.8±0.9 and 18.1±1.3 at 0, 2 and 4 hour post thaw, respectively. Percentage of intact plasma membrane was observed 70.5±1.4, 52.9±1.0 and 40.0±1.6 at 0, 2 and 4 hour post thaw, respectively. The percentage of spermatozoa with normal acrosomes was 79.1±0.4, 75.1±0.2 and 71.5±0.2 at 0, 2 and 4 hour post thaw, respectively.

DISCUSSION

It is now well recognized that epididymal spermatozoa harvested from the threatened dead animal can be cryopreserved and used for the production of viable embryos for the propagation of species (Andrabi *et al*., 2008). Cryopreservation is a

tool which offers an opportunity to preserve the germplasm from the dead animal either from domestic or non-domestic species. Sahiwal is well known local breed of South Asia whose population is drastically decreasing (Dahlin *et al*., 1998) because of crossbreeding with exotic dairy and beef breeds. It is shocking that crossbred animals are having poor adaptability to the indigenous stressful environment (Rehman *et al*., 2006; Garcia *et al*., 2003; Joshi *et al*., 2001).

Table I.- Post thaw characteristics (Mean±SE) of Sahiwal bull epididymal spermatozoa cryopreserved in tris-citric egg yolk extender at 0, 2 and 4 hour of incubation at 37°C.

Post thaw hour	Sperm characteristics (%)		
	Motility	Plasma Membrane integrity	Normal Apical Ridge
0	50.6 ± 1.5	70.5 ± 1.4	79.1 ± 0.4
2	33.8 ± 0.9	52.9 ± 1.0	75.1 ± 0.2
4	18.1 ± 1.3	40.0 ± 1.6	71.5 ± 0.2

Germplasm of Sahiwal breed is precious because of its productivity, adaptability and resistance against diseases in local harsh conditions. As population of Sahiwal breed is decreasing rapidly there is a need to adopt suitable approaches for its conservation. Cryopreservation technique is of choice if Sahiwal bull of high genetic value becomes dead accidentally to preserve its germplasm for IVF, in vitro embryo production or artificial insemination programme. In present study, routinely used tris-citric egg yolk cryodiluter was evaluated for its suitability to cryopreserve Sahiwal bull epididymal spermatozoa. The present study indicated that the use of tris-citric egg yolk cryodiluter effectively preserved the motility, plasma membrane and morphology of Sahiwal bull epididymal spermatozoa. The similar findings were observed in bovines (Goovaerts *et al*., 2006; Martins *et al*., 2007).

It is concluded that tris-citric egg yolk cryodiluter may be used for the cryopreservation of Sahiwal bull epididymal spermatozoa.

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