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Effect of cholesterol loaded cyclodextrin supplementation on tyrosine phosphorylation and apoptosis like changes in frozen thawed *Hariana* bull spermatozoa

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ABSTRACT

The beneficial effects of cholesterol loaded cyclodextrin (CLC) addition were evaluated in cryopreserved bull semen. Forty ejaculates were collected from Hariana bulls (n = 4), pooled and divided into 4 aliquots. All the aliquots were initially diluted in to egg yolk tris citrate and supplemented with CLC @ 0.5 mg (Group-II), 1.0 mg (G-III) and 2.0 mg (G-IV) CLC/120 × 10^6 spermatozoa or without CLC (G-I) that served as control. Extended semen was cryopreserved at −196 °C for 24 h. Seminal attributes like motility, viability, cryocapacitation like changes, tyrosine phosphorylation, apoptosis like changes in terms of mitochondrial transmembrane potential and DNA integrity were evaluated after equilibration and thawing. Results showed a significant increase in the motility, viability and acrosome intact spermatozoa in Group II as compared to other three groups. Further, the proportion of spermatozoa showing capacitation and acrosome reaction was also decreased ($P < 0.05$) significantly in Group II as compared to Group I, III, and IV. Immunoblot demonstrated a 32 kDa (p32) protein showing differential variation in the band intensity in all the four groups being lower in Group II. Further, the immunolocalization study revealed positive immune reactivity for tyrosine phosphorylated proteins at middle piece and neck (high fluorescence), post-acrosomal region (medium fluorescence), and principal piece (low fluorescence) of spermatozoa. Addition of CLC significantly increased ($P < 0.05$) the percentage of spermatozoa showing high transmembrane mitochondrial potential, and also, CLC @ 0.5 mg/120 × 10^6 in semen extender significantly decreased ($P < 0.05$) spermatozoa showing fragmented DNA after thawing as compared to control. Results of the present study indicate beneficial effects of CLC supplementation on cryodamage of spermatozoa by reducing the cryocapacitation and apoptosis like changes.

1. Introduction

Cryopreservation induces a number of structural and functional changes in bovine spermatozoa and most of those are irreversible in nature [1]. Changes in cell membrane like membrane destabilization, loss of cholesterol, decrease in membrane fluidity, altered membrane permeability and decreased osmotic resistance lead to cryocapacitation, acrosome reaction and apoptosis like changes causing decreased competence of spermatozoa to undergo successful fertilization. Among the various proposed mechanisms [1–3], loss of membrane cholesterol plays a predominant role in inducing cryocapacitation and apoptosis in cryopreserved bovine spermatozoa.

The ratio between cholesterol (C) and phospholipids (P) maintains membrane fluidity and integrity of a cell [3,5–7]. It plays

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critical role in sperm cryopreservation. Bovine spermatozoa shows a C: P ratio of 0.38 indicating its precautious role in maintaining lipid behavior and phase transitions during freezing and thawing [4]. Cryopreservation targets primarily the membrane cholesterol causing reduction in C: P ratio, and thereby results in loss of functional and structural dynamics of the membrane. Unlike fresh, bull spermatozoa show about 45% reduction in C: P ratio during pre-freezing stage and 60% loss after freezing and thawing states [8]. In swine about 50% and in stallion 28% cholesterol is reported to be lost during cryopreservation [9–11].

Cryocapacitation occurs due to loss of membrane cholesterol which makes the sperm less viable. Due to the critical role of C:P ratio in sperm cryopreservation, many strategies have been developed so far to increase the cholesterol content of the sperm membrane prior to freezing. This reduced the cryocapacitation like changes and improved the sperm cryosurvival [11,12]. Cryopreservation induces cryocapacitation and apoptosis like changes in spermatozoa leading to loss of spermatozoa competence [24–26]. Tyrosine phophorylation serves as one of the hall marks of capacitation and many tyrosine phosphorylated proteins during capacitation have been reported in literature in different species of animals [11,27]. Freeze-thaw cycles induce programmed cell death in spermatozoa through activation of caspases, reduction in the mitochondria transmembrane potential, fragmentation of sperm DNA and thereby decrease the functional competence of spermatozoa [24,25]. The loss of sperm membrane cholesterol has been implicated either directly or indirectly in all the above said events during freezing and thawing.

Cyclodextrins, -cyclic oligosaccharide sugars are able to incorporate the cholesterol into sperm cell membrane and thus emerged as one of the most convenient way of incorporation of cholesterol to the sperm cell membrane [7]. They have been used as a suitable vehicle for incorporation of cholesterol to the plasma membrane and increased cholesterol content of sperm cells in a number of species including boar [7,13–16], buffalo [17], bull [5,12,18–20], ram [21] and stallion [10,22,23] during cryopreservation.

Previous studies indicated the beneficial and protective effects of CLC supplementation on plasma membrane integrity, viability as well as acrosome integrity [4,12]. However, to the best of our knowledge, information pertaining to the effect of CLC on tyrosine phosphorylation of proteins and apoptosis like changes in sperm cells are meager. Therefore, in the present study, we attempted to address the effect of CLC on frozen and thawed bull sperm cells in terms of protein tyrosine phosphorylation and apoptosis like changes in terms of transmembrane mitochondrial potential and DNA integrity.

2. Materials and methods

All chemicals were purchased from Sigma Aldrich, St Louis, MO, USA unless or otherwise stated. Chemicals were cell culture/molecular biology grade. Ethical permission was taken from Institutional Animal Ethics Committee for collection of semen from bulls using artificial vagina (AV).

2.1. Animal selection

Four adult Hariana (*Bos indicus*) bulls, aged between 6.5 and 7.5 years, and weighing between 450 and -500 kg were reared under semi-intensive system of animal rearing at the Instructional Livestock farm of the University were selected for the study. They were regularly in use for the collection of semen in a schedule of twice per week. Bulls were apparently healthy and vaccinated as per the standard schedule of the Institute farm. Semen samples were collected twice a week during the month of February to May, and immediately after collection, the semen samples were transferred to the laboratory for further processing. Ejaculates containing higher than 80% motile spermatozoa were qualified only for the study.

2.2. Preparation of cholesterol loaded Methyl-β -cyclodextrin

Methyl-β -cyclodextrin was loaded with cholesterol as described earlier [12]. In brief, 200 mg of cholesterol was dissolved in 1 ml of chloroform and 1 gm of methyl-β -cyclodextrin was dissolved in 2 ml of methanol. A 0.45 ml aliquot of cholesterol was added to the cyclodextrin, and the mixture was stirred until the mixture appeared clear. The solution was then poured into a glass petridish; the solvents were removed and allowed to dry for 24 h. The resulted crystals were removed from the dish and stored in a glass container at room temperature. A working solution of CLC was prepared by adding 50 mg of CLC to 1 ml of Tris diluent (250 m*M* tris-hydroxymethyl aminomethane, 83 m*M* citric acid monohydrate, 69 m*M* D-(+)-glucose, pH = 7.0, 300 mOsm) at 37 °C and mixing of the solution was carried out using a vortex mixer.

2.3. Semen processing (initial evaluation and cryopreservation)

Semen was initially diluted in Tris diluents without egg yolk and glycerol to obtain final concentration of 120×10^6 spermatozoa. The diluted semen was divided into following 4 groups (one control and three treatments groups). The control group was without CLC (Group I); and treatment groups were containing 0.5 mg (group II), 1.0 mg (group III) and 2.0 mg (group IV) CLC/120×10^6 spermatozoa. Aliquots of all the four groups were incubated at 37 °C for 15 min for optimal incorporation of CLC to sperm membrane as described earlier [12]. Following incubation, each sample was diluted in Tris diluents containing 20% egg yolk and 7% glycerol maintaining the sperm concentration 80×10^6 cells/ml in each sample.

2.4. Semen cryopreservation

The extended semen samples were equilibrated at 4 °C for 4 h followed by cryopreservation using a biological cell freezer (IMV Technology, France) as per the standard protocol (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) as developed in the Semen Biology Laboratory, DUVASU, Mathura, India.

2.5. Evaluation of semen

2.5.1. Sperm motility and viability

Semen samples were evaluated at pre-freeze (after equilibration) and post-thaw (at 24 h after cryopreservation). Forward progressive sperm motility was evaluated on an automated thermostatic stage maintaining temperature of 37 °C using phase contrast microscope. Subjective assessment of motility was carried out by individual person to avoid individual intra laboratory variations in determination of motility. The motility pattern of sperm samples was done as described earlier [28–30]. In brief, rapid progressive motile spermatozoa were taken as “A” grade, slow progressive motile spermatozoa as “B” grade, non progressive motile spermatozoa as “C” grade and non motile spermatozoa were taken as “D” grade. Progressive motility (A + B), non-progressive motility (C) and non-motile (D) were calculated as a per cent of total (A + B + C + D). During the study, 40 replicates were collected and (A + B) were determined for the evaluation of sperm progressive motility. Sperm viability was evaluated by employing sensitive propidium iodide staining of spermatozoa using a fluorescent microscope. We also used simple Eosin-Nigrosin staining