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A 31-kDa seminal plasma heparin–binding protein reduces cold shock stress during cryopreservation of cross-bred cattle bull semen

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ABSTRACT

In the present study, a 31-kDa protein, purified from cattle bull seminal plasma heparin–binding proteins (SP-HBP), was characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and mass spectrometry. Raw semen of six cross-bred bulls was treated with 31-kDa HBP before cryopreservation to observe its effect on motility, viability, hypo-osmotic swelling test, acrosome integrity, *in vitro* capacitation/acrosome reaction, and oxidative stress at pre-freeze and frozen–thawed phases of cryopreservation. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of 31-kDa protein eluted and purified from SP-HBP (separated on acrylamide gels) resulted in a single band of 40 kDa. In matrix-assisted laser desorption/ionization–time of flight analysis, 12 peptides were identified with matching significantly ($P < 0.05$) to interlukin-6 of bovine with a top score of 55. Addition of 25 μg/mL of fluorescein isothiocyanate–conjugated 31-kDa protein to raw semen and incubation at 37 °C for 20 minutes before cryopreservation resulted in its binding mainly to head region. Treatment of semen with 31-kDa HBP resulted in a significant ($P < 0.05$) average increase of 9.2%, 6.8%, and 11.7% and 5.5%, 6.5%, and 11.0% in motile, viable, hypo-osmotic swelling–responsive spermatozoa in six bulls at pre-freeze and frozen–thawed phases of cryopreservation, respectively. Percentage of spermatozoa with intact acrosomes nonsignificantly enhanced in the semen treated with 31-kDa HBP at both phases of cryopreservation. An average nonsignificant increase of 3.1% in *in vitro* capacitated and acrosome-reacted spermatozoa was obtained in semen supplemented with 31-kDa HBP. Addition of 31-kDa HBP also nonsignificantly reduced Malonadialdehyde (MDA) level by 10.7 and 19.3 μM/10^9 spermatozoa in prefrozen and frozen–thawed semen, respectively. The results obtained here indicate to conclude that treatment of cross-bred cattle bull semen with 31-kDa HBP protects the spermatozoa from cold shock effect by coating the sperm surface.

1. Introduction

Cold shock is referred as the stress response shown by spermatozoa as a reaction to a drop in temperature. Generally, cold shock damage manifests itself as a decrease in cell metabolism, altered membrane permeability, loss of intracellular components, irreversible loss of sperm motility, and an increase in the number of dead spermatozoa. The cellular damage resulting from cooling or freezing affects both the structure and function of the cells. Induction of premature acrosome reaction, altered mitochondrial function, reduction of motility, and failure of chromatin decondensation, all of which influence the viability and fertility of the sperm cells have been reported

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by different authors [1–4]. Amman and Graham [5] and Lessard et al. [6] observed cooling as a major stressor, as a result of which membrane-bound phospholipids reorient themselves into a different configuration that disrupt membrane functions and permeability. Andrabi [7] suggested that, even under ideal conditions, it is inevitable that some damage will occur to spermatozoa during the freezing process. Lessard et al. [6] were of the opinion that sperm viability decreased by 50%, whereas fertilizing capacity was affected by a factor of sevenfold after cryopreservation. Chilling/freezing has some adverse effects on the spermatozoa manifested as depression in viability, structural integrity, depressed motility, and conception rate [1,8,9]. The acrosome membrane suffered most from cold shock that accounts for most of the fertility failure [10]. Increased level of reactive oxygen species during cryopreservation have also been correlated with decreased sperm motility [11], increased DNA sperm damage [12], sperm cellular membrane lipid peroxidation [13], and decreased efficacy of oocyte–sperm fusion [14]. Several studies have focused on identifying parameters of damage during freezing/thawing, tests to screen sperm quality of frozen–thawed semen, evaluation of alternative cryoprotectants, other additives, and freezing procedures to improve sperm viability and fertility [15,16]. Most of the progress in improving survival of frozen–thawed spermatozoa focuses on minimizing the oxidative damage and decreasing the osmotic stress on spermatozoa.

Seminal plasma contains factors that modulate the fertilizing ability of sperm [17]. In most mammals, the sperm must reside in the female reproductive tract for about 6 to 8 hours to undergo capacitation. The final step of capacitation, the "acrosome reaction," involves erosion of the acrosome and release of its enzyme contents. The sperms are held in the oviductal reservoir by binding to the epithelium through Binder sperm proteins (BSPs) on the head of bull sperm before undergoing capacitation [18]. The BSPs are produced by the bovine seminal vesicles and represent approximately 70% of the total protein content of bovine seminal plasma [19]. Three BSPs, BSP-1 (BSP-A1/A2), BSP-3 (BSP-A3), and BSP-5 (BSP-30 kDa), are implicated in binding the sperm to the oviductal epithelium and adsorb to the sperm surface when the sperm come in contact with vesicular secretions in the seminal plasma [20] Loss of BSP-1 from sperm has been associated with the removal of cholesterol from the sperm plasma membrane during capacitation [21]. Three major classes of heparin-binding protein (HBPs), namely 14 to 27 kDa (spermadhesins), 24 kDa (cysteine-rich secretory proteins), and 31 kDa (Fn-II type proteins), in seminal plasma of bovines that interact with the sperm surface and participate in sperm function have been also reported [22,23]. The HBPs are produced by male accessory sex glands, secreted into seminal fluid, and on ejaculation bind to the sperm. They are more abundant on the surface of ejaculated spermatozoa than on the plasma membrane of epididymal spermatozoa [22]. The HBPs with molecular weight 18 to 55 kDa were named as fertility associated antigens (FAA) [17]. The HBPs bind to sperm membrane choline phospholipids and capacitation factors, namely heparin and glycosaminoglycans, at ejaculation [20] resulting in efflux of phosphatidylcholine and cholesterol which is an important step during capacitation, acrosome reaction, sperm–oocyte fusion, and fertilization [24,25]. Addition of HBPs to epididymal sperm induced heparin-stimulated capacitation/acrosome reaction [26]. Many diverse proteins, especially seminal plasma heparin–binding proteins (SP-HBPs), are also being tested to affect increased fertilization capability of frozen–thawed sperm [27–30]. Therefore, the present study was conducted with the following objectives:

- Purification and characterization of 31-kDa SP-HBP from cross-bred cattle bull seminal plasma.
- To observe the effect of 31-kDa SP-HBP on sperm attributes during different stages of cryopreservation.

2. Material and methods

Experiment was conducted on cattle bull spermatozoa to observe the effect of 31-kDa HBP treatment on sperm parameters during the process of cryopreservation.

2.1. Procurement of semen samples and chemicals

Three ejaculates from each of six healthy HF cross cattle bulls collected by artificial vagina were procured from GADVASU, Dairy Farm and Semen bank, Bhattian, Khanna, Ludhiana, India. A good-quality fresh ejaculate (1–2 mL) with minimum initial motility of 60% or more and mass activity of 3+ to 4+ and abnormal morphology of 25% or less of each bull were processed for cryopreservation. Seminal plasma was separated by centrifuging pooled semen of six bulls at 3360 × *g* for 5 minutes. Pooled seminal plasma was re-centrifuged at 11,200 × *g* for 10 minutes to obtain sperm-free seminal plasma and was stored at −20 °C till use. All required chemicals were procured from Sisco Research Laboratories and Sigma-Aldrich.

2.2. Purification of 31-kDa protein from affinity-purified SP-HBP

HBPs were purified from seminal plasma containing cocktail protease inhibitors (10 μL/mL) by affinity chromatography (Spectrum) as explained in our previous study [31]. Purified HBPs were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; Cleveres Scientific Company). Graphic image of purified HBPs and its separation by SDS-PAGE are shown in Figures 1 and 2, respectively. A well, cut from the gel, was stained with coomassie brilliant blue, and rest of the gel was stored at 4 °C till the bands appear in the stained well. Then stained well was aligned with the rest of the unstained gel and strip corresponding to 31 kDa was cut with a sharp blade. The strip was washed three times (5 minutes each) with 2 mL of 250 mM Tris buffer/250 mM EDTA, pH 7.4, in a tube followed by rinsing three times with double distilled water. Water was removed, and the gels were chopped; 20 mM Tris buffer, pH 7.4, containing 0.1% SDS was added to the gel in the ratio of 2:1 and was sonicated for 3 minutes in ice bath (5–6 pulses of 30 seconds). For