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Loss of heat shock protein 70 from apical region of buffalo (*Bubalus bubalis*) sperm head after freezing and thawing

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ABSTRACT

The post-thaw fertility of frozen-thawed mammalian spermatozoa is substantially low as compared with that of fresh sperm. Furthermore, the post-thaw fertility of the cryopreserved buffalo sperm has been reported to be poor as compared with that of cattle sperm. Recently, heat shock protein 70 (HSP70) has been found to play a critical role in mammalian fertilization and early embryonic development in boar and cattle. However, the presence of such fertility-related HSP70 in buffalo sperm and its status after cryopreservation has not been reported so far. Thus, a study was conducted to determine the effect of cryopreservation on the level and distribution pattern of HSP70 molecule in buffalo sperm after cryopreservation. Buffalo semen samples, after dilution in semen extender, were aliquoted in straws and divided into two groups. One group was not cryopreserved, and the other group was cryopreserved for 60 days. Sperm proteins were extracted from both non-cryopreserved (NC) and cryopreserved (C) sperm and subjected to Western blot analysis for detection of HSP70 using a monoclonal anti-HSP70 antibody. The distribution pattern of these proteins in buffalo sperm was also monitored before and after cryopreservation using indirect immunofluorescence technique. A prominent 70-kDa protein band of HSP70 protein was detected in protein extracts of both NC and C buffalo sperm. Densitometry analysis revealed that the intensity of 70-kDa HSP70 protein band of cryopreserved sperm decreased significantly ($P < 0.05$) compared with that of NC sperm. However, the level of HSP70 in cryopreserved extended seminal plasma (ESP) did not change as compared with that of NC samples indicating a possible degradation of HSP70 in the spermatozoa itself rather than leakage of the protein into the ESP. Furthermore, Western blot also confirmed that several HSP70 immunoreactive protein bands detected in the ESP were contributed by the egg yolk that was added to the extender. Immunocytochemistry revealed that HSP70 proteins were distributed over the apical region of sperm head and/or acrosome, post-acrosomal, and middle piece regions of NC buffalo spermatozoa. However, the fluorescence signal of apical region of sperm head was lost significantly ($P < 0.05$) after a cycle of freezing and thawing. Thus, the present study confirmed that there was loss of HSP70 from buffalo sperm head after freezing and thawing of buffalo spermatozoa, and this may be one of the causes of the reduced post-thaw fertility of sperm in this species.

1. Introduction

Semen cryopreservation is an indispensable tool to preserve and propagate elite germplasm for breeding and improvement of farm animal species through artificial insemination. However, the post-thaw motility and thus the fertility of cryopreserved semen is found to be reduced

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substantially making it a major impeder of success of the artificial insemination [1,2]. Even the post-thaw motility of the cryopreserved buffalo sperm has been reported to be significantly reduced as compared with that of fresh sperm [3]. Although the sperm structure and seminal plasma (SP) composition varies substantially across the species, the composition of semen extender used for diluting semen and protocols used for subsequent cryopreservation remains primarily similar in all the species leading to variation in the extent of cryodamage and success of artificial insemination in different species [1,3]. The post-thaw motility loss and the extent of cryodamage have been observed to be higher in buffalo spermatozoa as compared with those of cattle [3]. Cryopreservation of semen has been found to be associated with sublethal and/or lethal damage of sperm including irreversible rupture of plasma membrane, leakage and denaturation of proteins and enzymes, lipid peroxidation leading to oxidative stress, DNA damage, loss of sperm viability, and functional ability of the surviving population [1,4,5].

Mammalian heat shock protein 70 (HSP70), although first identified as stress inducible protein [6], has also been reported to play role in several functions of molecular chaperones in mammalian cells including protein folding and refolding, recognition of non-native protein conformation, transmembrane transport of proteins, prevention of aggregation of newly synthesized proteins, disposal of damaged and/or defective proteins, targeting misfolded proteins to the proteasome and so forth [7,8]. This 70-kDa heat shock protein is known to exist in two forms, the constitutive form also known as the heat shock cognate protein 70 (HSC70) and the inducible form (HSP70) [9]. The HSC70 is present in cells under normal conditions and has many important housekeeping functions and is expressed constitutively in the lens of Zebrafish eyes [10] and chick embryos [11]. The inducible forms are only expressed under stress conditions, which assist folding of newly synthesized polypeptide chain and carry out the repair and degradation of altered or denatured proteins. In normal or unstressed cells, inducible HSP70 are not expressed but the constitutive forms may be present.

In recent years, HSP70 protein has been reported to have role in male fertility in mice [12]. In both boar [13] and bovine [14] spermatozoa, the re-localization and redistribution of HSP70 from the acrosomal area to the equatorial segment and post-acrosomal regions during induced capacitation and acrosome reaction suggested a potential role of these molecules during fertilization. Consistently, an anti-HSP70 antibody has been found to reduce the bovine and swine fertilization rate *in vitro* in a dose-dependent manner [13,15]. Recently, heat shock protein 70 kDa protein 2 has been suggested to play role in human sperm–egg recognition [16,17]. The addition of heat shock protein A8 (HSPA8, formerly known as heat shock cognate protein 73 kDa [HSC70]) to the cryoprotective media improved the survival of brown bear spermatozoa during chilling and after cryopreservation [18]. Thus, it appears that HSP70 of sperm has a significant physiological role in fertilization. Cryopreservation of sperm is associated with reduced fertilizing potential; no reports are available regarding the status of this fertility-related HSP70 protein in cryopreserved sperm of any ruminant species. Hence, in the present study, an attempt was made to determine the effect of cryopreservation on the status of HSP70 molecule in buffalo sperm and the distribution pattern of these proteins in sperm after cryopreservation. Such an understanding of the molecular effects of cryopreservation on these proteins of sperm could suggest methods for improving fertility of frozen and/or thawed buffalo semen.

2. Materials and methods

Four healthy Murrah buffalo (*Bubalus bubalis*) bulls (3–5 years of age) with good body condition were maintained under uniform feeding and management regimen at Nandini Sperm Station, Hesaraghatta, Bangalore. All the experimental protocols of the study were carried out following the Institutional Animal Ethics Committee guidelines. Semen was collected twice a week from each bull using an artificial vagina (IMV Technologies, L'Aigle, France) maintained at 40 °C. In a day, two ejaculates were collected from each bull after an interval of approximately 15 minutes, and both the ejaculates of the same bull were pooled, and this constituted one semen sample. Unless otherwise specified, all the chemicals and/or reagents used in the study were of molecular biology grade and obtained from either Sigma-Aldrich Co. (MO, USA) or Sisco Research Laboratories (Mumbai, India).

2.1. Collection and cryopreservation of buffalo semen

Four semen samples were collected from four different bulls. Immediately after collection, semen samples were evaluated for their mass activities by light microscopy at 10× magnification. The semen having mass activity of +3.0 and above (in a scale of 0–4.0) were used in the study. Within 5 to 10 minutes of collection, a small aliquot of semen (0.5 mL, approx. 600–700 million sperm) was centrifuged at 275 × *g* for 10 minutes at room temperature to separate fresh seminal plasma (SP) and sperm pellet. The proteins from the sperm pellet were extracted as per the method described in the following section. These sperm protein extracts (SEs) and SPs were considered as fresh SE and SP. These fresh samples were stored at −20 °C until Western analysis of cryopreserved samples. The other major aliquots of the semen samples were subjected to freezing protocols as described in the following section. Semen sample from each bull was extended in a Tris-citrate-fructose–glycerol-egg yolk extender (0.2 M Tris, 0.065 M citric acid monohydrate, 0.055 M fructose, 7% [v:v] glycerol, 20% [v:v] egg yolk, benzyl penicillin [1,000,000 international units/L] and streptomycin sulfate [1 g/L]). Spermatozoa concentration in extender was adjusted to 80×10^6 cells/mL. The extended semen was filled in 0.25-mL straws (IMV Technologies, L'Aigle, France), and these were sealed and labeled for bull number and date of collection. For each bull, about 75 straws were prepared. Of these, 30 straws were processed for separation of sperm pellet and semen extender. Thus, these semen straws not subjected to cryopreservation procedure were considered as non-cryopreserved (NC) samples. The samples pertaining to fresh SE and SP, extended but NC SE and extended seminal