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Species-specific and differential expression of BSP-5 and other BSP variants in normozoospermic and asthenozoospermic buffalo (*Bubalus bubalis*) and cattle (*Bos taurus*) seminal plasma

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ABSTRACT

Binder of sperm-5 (BSP-5) is one of the fertility-associated proteins of cattle seminal plasma. Binding of sperm to the oviductal epithelium is mediated by BSP group of proteins. However, it is not clear, whether this protein is also involved in sperm motility. In the present study, attempts were made to characterize BSP-5 protein in both normozoospermic (NS) and asthenozoospermic (AS) Murrah buffalo (n = 18; *Bubalus bubalis*), Holstein Friesian (n = 8, *Bos taurus*) and Jersey cattle (n = 8; *Bos taurus*) bull seminal plasma and also study its expression pattern in these species. 1-D Western blot demonstrated three major BSP-5 immunoreactive protein bands (24.2 kDa, 20.5 kDa, and 12.3 kDa) in buffalo seminal plasma. Of these, the intensities of 24.2 and 20.5 kDa protein bands reduced significantly ($P \leq 0.05$) in seminal plasma of AS group compared to that of NS group. On the contrary, the expression of 12.3 kDa protein band did not vary significantly between the groups. In Holstein Friesian seminal plasma, at least six BSP-5 immunoreactive protein bands (25.1, 23.6, 19.5, 13.8, 13.1 and 12.3 kDa) could be detected. Of these, the intensities of 23.6, 13.8/13.1 and 12.3 kDa protein bands decreased ($P = 0.058, 0.111, 0.053$) in AS group bulls compared to NS bulls. Holstein Friesian bull seminal plasma demonstrated a BSP-5 immunoreactive duplex protein band of 13.8/13.1 kDa, which was not evident in buffalo seminal plasma. In 2-D Western blot, a train of five BSP-5 immunoreactive duplex protein spots (Mr 21.0–27.6 kDa, pI of ~3.9–5.1) was detected. Mass spectrometry of one of the representative duplex spot confirmed that these were BSP-5 and BSP-3 proteins, respectively. Indirect immunofluorescence studies showed that BSP-5 is primarily localized to the mid-piece/mitochondrial region of buffalo spermatozoa. To conclude, the findings of the present study could establish the significance and association of BSP-5 proteins in sperm motility and how their level differ in semen from two different clinical groups of buffalo bull (NS vs. AS). Further, the study also demonstrated that the expression pattern of BSP-5 and other BSP variants in seminal plasma of bulls is species-specific.

1. Introduction

Mammalian fertilization is an intricate process involving the fusion of a motile sperm and a reproductively mature egg and sperm motility is a key factor responsible for male fertility and in turn successful fertilization. In domestic animals, about half of the unsuccessful fertilization is attributed to infertility of bulls. Among the buffalo and cattle bulls, a substantial number suffer from low sperm motility, a condition clinically known as asthenozoospermia. Moreover, the average percentage of progressive motility of frozen-thawed buffalo spermatozoa is substantially low as compared to that of cattle sperm [1,2]. However, the precise reason and mechanism for this reduced progressive motility of buffalo sperm are not known. Hence, there is a desperate need to decipher the functional components that are associated with motility of buffalo sperm.

Over the few former decades, seminal plasma proteins and their relevance to sperm functions have become a prime area of study [3]. Seminal plasma is a concoction of secretions contributed by the testis, epididymis, and accessory sex glands, operating as a vital medium for sperm survival and functions post

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ejaculation [4]. Several studies have demonstrated the influence of these seminal plasma components on fertility of bulls [5–9]. Manjunath et al. [10] hypothesized that certain components of seminal plasma can notably affect sperm function by binding to spermatozoa. Binder of Sperm (BSP) proteins is one such superfamily of proteins that play an important role in sperm maturation events and are highly studied in regard to their biochemical, structural and functional aspects [3]. BSP family of proteins exist in multiple forms in mammalian seminal plasma and are ubiquitous in nature [11]. BSPs that were purified from bovine seminal plasma include BSP-1, BSP-3, and BSP-5 (earlier nomenclatured as BSP-A1/A2 or PDC-109, BSP-A3, and BSP-30-kDa, respectively) [3]. BSP-1 and BSP-5 are glycoproteins containing galactosamine, sialic acid, and neutral sugars, whereas BSP-3 is not a glycosylated member [12–15]. The molecular mass (Mr) of BSP-5 proteins falls in the range of 28–30 kDa whereas that of BSP-1 and BSP-3 proteins falls in the range of 15–16 kDa [13,16]. BSP proteins have been demonstrated to cater regulatory functions like decapacitation of sperm and stabilization of the sperm membrane leading to capacitation induced by glycosaminoglycans and high-density lipoproteins. Upon ejaculation, the sperm are coated with BSP proteins by interacting with specific membrane phospholipids. Eventually, this leads to decapacitation of spermatozoa and then while travelling through the female reproductive tract BSP proteins consequently encounters high-density lipoproteins which ultimately triggers the process of capacitation [10]. The homologues of BSP proteins have been identified in stallion, boar, goat, bison and ram seminal plasma [11,17–21]. Additionally, BSP proteins aid in the binding of sperm to the bovine oviductal epithelium and also in the formation of oviductal sperm reservoir [22,23]. Recently, it was reported that BSP coating on the sperm is increased during freezing and may partly reduce the fertility of cryopreserved bovine semen [24].

Incorporation of PDC-109 (BSP A1/A2) at 2 μM concentration in bovine sperm culture in vitro significantly increased sperm motility as compared to the untreated group [25]. The levels of BSP-30-kDa protein in the accessory sex gland fluids of Holstein bulls demonstrated a quadratic association with their fertility indexes [8]. Hence, the significance of BSP proteins in sperm functions is highly appreciable. However, to the best of our knowledge, the specific role and the association of BSP-5 proteins with sperm motility is not explored so far in any ruminant species. Hence, it is hypothesized that the expression pattern of BSP-5 protein may vary between the seminal plasma from normozoospermic (NS) and asthenozoospermic (AS) group of buffalo and cattle bulls. Thus, in this study, attempts were made to characterize the BSP-5 and its molecular forms, if any, in buffalo and cattle seminal plasma in the first place and also find the association of BSP-5 protein with sperm motility in normozoospermic (NS) and asthenozoospermic (AS) buffaloes as well as related species such as Jersey (JY) and Holstein Friesian (HF) bulls. The results of the present investigations could provide us an insight into the specific role of BSP-5 in sperm motility of buffalo and other related species.

2. Materials and methods

Eighteen Murrah buffaloes (*Bubalus bubalis*), eight of each Jersey and eight Holstein Friesian (HF) cattle bulls, between 3 and 5 years of age, and 400–700 kg of body weight, were maintained with the same feeding and management regimen at Nandini Sperm Station (NSS), Hessarghatta, Bangalore, Karnataka, India. Semen samples were collected twice a week from each bull. All the experimental protocols of the study were carried out following the guidelines of Institutional Animal Ethics Committee.

2.1. Collection of buffalo semen, sperm motility analysis by computer-assisted semen analyzer (CASA) and its processing

Semen ejaculates from Murrah buffalo, Jersey and Holstein Friesian cattle bulls were collected using an artificial vagina (IMV, France) maintained at 40 °C. Shortly after collection, semen samples were assessed for their mass activities by light microscopy at 10× magnifications. A minor aliquot of semen sample was diluted (1:10) in sperm-Tyrode's albumin lactate pyruvate hepes buffer (sp-TALPH) media, pH 7.4 [100-mM NaCl, 3.1-mM KCl, 0.4-mM EDTA di-sodium salt, 0.4-mM $MgCl_2 \cdot 6H_2O$, 0.3-mM $NaH_2PO_4 \cdot 2H_2O$, 21.6-mM Sodium lactate, 2-mM $CaCl_2 \cdot 2H_2O$, 1-mM Sodium pyruvate, 40-mM hepes, 10-mM $NaHCO_3$ and 1 mg/mL (w/v) polyvinyl alcohol (PVA, 30–70 kDa)] and the progressive motility of sperm was evaluated by computer-assisted semen analyzer (CASA, version 3.2.0; Microptic, Barcelona, Spain) [26]. Based on the percentage of progressive motility as assessed by CASA, buffalo and cattle semen samples having progressive motility ≥70% (categorized as normozoospermic, NS) and progressive motility ≤ 40% (categorized as asthenozoospermic, AS) were only used in the study. Semen samples having progressive motility above 40% and below 70% were excluded from the study so as to have a clear-cut difference in target protein level between the NS and AS group of bulls.

The other major aliquot of semen was centrifuged (5810R, Eppendorf, Hamburg, Germany) at 275×*g* for 10 min at room temperature, to separate sperm pellet and seminal plasma as supernatant. The seminal plasma was again centrifuged at 20,800×*g* to separate traces of sperm and cellular debris, and the supernatant was collected, to which appropriate volume of protease inhibitor cocktail (PIC) (cOmplete; Roche Diagnostics GmbH, Mannheim, Germany) was added and stored as aliquots at −20 °C for subsequent analysis. The sperm pellet was washed thrice with sp-TALPH, pH 7.4 at 275 × g for 10 min and a minor aliquot of sperm pellet was re-suspended in sp-TALPH, pH 7.4 for use in immunolocalization technique.

2.2. Determination of protein concentration and sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The protein concentration in seminal plasma was determined by Bradford protein assay [27] using purified BSA (4 mg/ml) as standard (Fraction V, Sigma Chemicals Co, MO, USA). Seminal plasma of Murrah buffalo, Jersey and Holstein Friesian cattle bulls were resolved by 15% uniform SDS-PAGE according to the method of Laemmli [28]. The molecular mass of separated proteins was determined by using standard protein molecular weight markers (Precision Plus dual colour standards, Bio-Rad, Hercules, California, US).

2.3. Western blotting of Murrah buffalo, Jersey and Holstein Friesian seminal plasma proteins

Western blotting was performed for Murrah buffalo, Jersey and Holstein Friesian seminal plasma proteins for detection of BSP-5 proteins. The resolved proteins on the gel were transferred onto the Immobilon-P PVDF membrane (pore size, 0.45 mm) by a two-step transfer method [29]. The qualitative assessment of protein load and transfer efficiency was validated by ponceau S staining [30]. The unbound areas on the membrane were blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline with Tween-20, pH 7.6 [TBS-T; 20 mM Tris, 150 mM NaCl, 0.1% (v/v) Tween-20] in a refrigerator at 4 °C overnight. The blots were incubated with rabbit anti-bovine BSP-5 polyclonal antibody (11.4 μg/ml; a generous gift from Professor P. Manjunath, University of Montreal, Quebec,