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Original article

PROTEIN AND LIPID SPECIES IN SEMINAL PLASMA OF FERTILE HOLSTEIN-FRIESIAN BULLS

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Summary

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Protein and lipid molecules in seminal plasma (SP) collected from fertile bulls were investigated. Semen was collected from 10 bulls (2 ejaculates each) and examined for standard semen analysis. Raw SP was recovered by centrifugation and total protein (TP) concentration was determined using a refractometer. Raw SP was desalted using a Sephadex G-25 desalting column then both raw and desalted SP was subjected to SDS-PAGE. Neutral lipids and phospholipids of raw and desalted SP were separated by thin-layer chromatography (TLC). The results revealed that, all bulls had normal semen characteristics and TP concentration in SP ranged from 7.0 to 10.4 g/dL except bull No. 6 had a relatively low concentration of 4.9 to 6.8 g/dL. Neither proteins nor lipids species were different between raw and desalted SP. Seventeen proteins were detected ranging from 8.5 to 185.8 kDa, and those of 12, 13.5, 15, 21, 23 and 38 kDa were predominant. Notably, proteins of 10, 17.5, 19, 21, 80 and 185.8 kDa might be new candidates of SP proteins (SPPs). The detected neutral lipid spots corresponded to cholesterol, 1,2-dimyristoyl glycerol, 1,2-dioleoyloglycerol, 1,3-dimyristoyl glycerol and 1,3-dioleoyloglycerol. The detected phospholipids spots corresponded to non-migrating phospholipids, sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), cerebroside and polyglycerol phosphatide. Cholesterol represents the major molecule of neutral lipids, whereas SM, PC, PI and PE represent the major phospholipids. Noteworthy, there were 2 species of diacylglycerol (DAG) and 3 species of PI in bovine SP. In conclusion, this study gave a general picture of SP protein and lipid species in fertile bull semen, which might serve as fundamental knowledge for either semen analysis or prediction of male fertility.

Key words: cholesterol, desalted SP, fertility, Holstein-Friesian bulls, SP proteins

INTRODUCTION

Evaluation of semen quality is based on both macroscopic (volume, colour, odour, and consistency) and microscopic (motility, morphology, and concentration) examinations. However, studies have shown such evaluation of semen quality to be inadequate, as semen shown high quality by these examinations may be subfertile. Therefore, determination of additional biochemical parameters has been proposed (Argov-Argaman *et al.*, 2007; Brinsko *et al.*, 2007; Chacur, 2012). Sperm structure and fertilising ability depend upon lipid composition of both sperm plasma membrane and seminal plasma (SP). The role of cholesterol in sperm cell function has been demonstrated in numerous studies which aimed to improve the sperm cryopreservation technique and composition of semen extenders (Bailey *et al.*, 2008; Beer-Ljubić *et al.*, 2009). Cholesterol has been added to diluents to prevent freeze-thaw damage of sperm cell, which may impair sperm cell motility and fertility (Cerolini *et al.*, 2001; Janett *et al.*, 2003). Besides cholesterol, SP also contains triacylglycerols, their oxidation serving to meet the sperm energy requirements.

It is commonly accepted that SP contains a variety of biochemical components such as proteins and lipids, which are relatively specific for the regulation of sperm function (Strzezek *et al.*, 1992) including those preserving sperm viability and interacting with the female genital tract during fertilisation (Töpfer-Petersen *et al.*, 2005). Indeed, SP proteins (SPPs) play important roles in the physiology and alterations of sperm cell in both male and female genital tracts. For instance, these SPPs are essential for sperm cell function particularly for their interactions with the various milieus of the female genital tract and the oocyte and its vestments (Rodríguez-Martínez *et al.*, 2011; Caballero *et al.*, 2012). Understanding the physiological functions and relationships between SPPs and sperm characteristics is an important step toward identifying potential biomarkers of male fertility, and in improving sperm cryopreservation.

Mammalian SP is a complex secretion mainly originating from the testes, epididymides and accessory sex glands and significantly contributes to semen volume. Despite its physiological significance as the carrier of spermatozoa to the female genital tract, the biochemical characteristics and physiological roles of the various SPPs and lipids are poorly understood. In bovine, the major protein fraction of SP (30–50 mg/mL) constitutes a family of closely related proteins named bovine SP protein (BSP)-A1, BSP-A2, BSP-A3 and BSP-30 kDa (Manjunath & Sairam, 1987). Previous studies elucidating the biological role of these abovementioned SPPs revealed that they are secreted by seminal vesicles (Manjunath *et al.*, 1987) and bind to the sperm surface at ejaculation (Manjunath *et al.*, 1994) where the binding sites for it on the sperm membrane are choline phospholipids (Desnoyers & Manjunath, 1992), specifically phosphatidylcholine (PC), PC-plasmalogen and sphingomyelin (SM). The first functional role identified for BSPs is that they promote the capacitation of bull sperm (Thèrien *et al.*, 2005). Moreover, two additional functions were proposed for BSPs. The first one is the activity of protein kinase C (PKC) and of tyrosine protein kinase (TPK) inhibited *in vitro* by BSP-A1/A2 (Yu *et al.*, 2003). It was proposed that the inhibition of PKC may serve to prevent the premature acrosome reaction of sperm in the female geni-

tal tract. The second function of BSPs is to mediate the binding of sperm to the oviductal epithelium (Gwathmey *et al.*, 2006), and being involved in prolonging sperm survival during storage and in maintaining sperm motility in the oviduct. Thus, identification of these proteins helps to explain the mechanisms underlying male fertility and infertility (Xiao-Yan & Jia-Hao, 2011).

Lipids are a basic component of semen, contributing to the membrane structure, metabolism of spermatozoa and their ability to capacitate and fertilise the female gamete (Mann & L-Mann, 1981). Traditionally lipids can be defined as polar compounds that are insoluble in water and may be enriched by the treatment/extraction with organic solvents such as chloroform or hexane (Christie & Han, 2010). Lipid and fatty acid composition is associated with semen characteristics, which is considered as important reproductive predictor. Cholesterol, the most abundant lipid molecule in the sperm membrane and SP is sensitive to peroxidation. Reduced cholesterol concentration in the membrane impairs membrane stability, resulting in premature deterioration of the sperm cells (Van Gestel *et al.*, 2005). Since, knowledge of the biochemical composition of SP is essential to improve storage conditions and to predict both the storage tolerance and fertilising ability of spermatozoa. Moreover, recently Almadaly *et al.* (2015) confirmed that desalted SP added to semen extender has beneficial effects on sperm cell function of frozen-thawed Japanese Black bull spermatozoa. Therefore, the current study aimed to identify protein and lipid molecules in raw and desalted SP of Holstein-Friesian bulls of proven fertility in trial to detect some biochemical markers of bull fertility and to improve fertility of frozen semen.

MATERIALS AND METHODS

Animals

Semen was collected from ten Holstein-Friesian bulls (2.5–5.5 years-old) during the winter season. These bulls were kept at Genetics Hokkaido Association (Hokkaido, Japan). Bulls were routinely ejaculated at the same interval once a week but to eliminate any potential differences in semen quality due to serial ejaculates, all samples were collected with an artificial vagina (AV) from all bulls, two ejaculates each with a minimum of 1 h interval. In total, 20 ejaculates were collected from 10 bulls. All bulls were of known proven fertility; these bulls had conception rate higher than 60% after artificial insemination. This study was approved by the Institutional Committee of Animal Experiments of Hokkaido University and all experiments were carried out in accordance with the Hokkaido University Animal Experimentation Regulations.

Semen collection and evaluation

Bulls were mounted on dummy teaser, and semen was collected into a disposable graduated tube using a warmed (38.5 ºC), sterile AV. Macroscopic evaluation of the collected ejaculates for colour, volume and hygienic quality was conducted immediately after collection. Motility was assessed at 400× magnification under a pre-warmed cover slip and the percentage forward progressive sperm (% progressive motility) estimated in increments of 5%. Sperm concentration in each ejaculate was estimated by haemocytometer (Graham, 1996). All semen characteristics (colour, volume, progressive motility and concentration) of the first and the second ejaculates were within normal values in all bulls.

Media

Electrophoresis sample buffer (4% SDS, 10% β-mercaptoethanol, 20% glycerol, pH 6.8), was used in electrophoresis of SPPs. Acrylamide was purchased from GE Healthcare (AB, Sweden) while methylene bisacrylamide was purchased from Amersham Biosciences (AB, Sweden). All electrophoresis chemicals were of analytical grade and procured from commercial suppliers, mostly Sigma-Aldrich (St. Louis, MO, USA) and Wako (Osaka, Japan). All solvents used for lipid extraction and separation were of analytical grade. Toluene, diethyl ether, 99.5% ethanol, conc. NH_3 (28%), isopropanol, methyl acetate, methanol, chloroform, KCl and sulfuric acid were purchased from Wako (Osaka, Japan). Silica gel $60F_{254}$ thin layer chromatography (TLC) plates (20 × 20) and silica gel 60 high performance TLC (HPTLC) plates (20 × 10) were purchased from Merck (Darmstadt, Germany). Lyso-phosphatidylcholine (lyso-PC); SM; L,α-PC; L,α-phosphatidylserine (PS); L,α-phosphatidylinositol (PI) and L,α-phosphatidylethanolamine (PE) were obtained from Sigma and used as reference phospholipid standards. Cholesterol, 1,2/1,3-dioleoylglycerol (1,2/1,3-DOG), 1,2-dimyristoyl-sn-glycerol (1,2-DMG) and 1,3-dimyristoyl-sn-glycerol (1,3-DMG) were purchased from Wako but 2-oleoylglycerol was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used as neutral lipids standards.

Recovery and desalting of SP

The collected semen of each bull ejaculate was separated into spermatozoa and SP by centrifugation at 3000 × g for 15 min at 5 ºC; the supernatant (SP) per each ejaculate was collected and transferred to the laboratory at 5 ºC. The collected supernatants were transferred into 1.5 mL vials and re-centrifuged at 12000×g for 30 min at 4 ºC to obtain on sperm-free SP. The collected SP (raw SP) was stored at 4 ºC during analysis and subsamples from each bull were dispensed before gel filtration for protein (60 µL), lipid (100 µL) and electrolytes (50 µL) analyses. An aliquot (5 µL) of raw SP was used for determination of total protein (TP) concentration (g/dL) of SP by using hand-held Refractometer (ATAGO, Brix 0-32%, Japan) according to Marsh and Fingerhut (1962). The collected SP from all bulls was pooled before (pooled raw SP) and after (pooled desalted SP) gel filtration and similarly aliquots were dispensed for protein, lipid and electrolytes analyses. Sodium (Na^+), potassium (K^+) and chloride (Cl^-) ions concentration in raw SP were determined by using automated equipment (Dri-Chem 800V; Fuji Film, Tokyo, Japan; lower limit of measurement, 1 mmol/L). Raw SP was desalted by using gravity protocol according to the manufacturer's instructions. Only 2.5 mL of raw SP obtained from each ejaculate was applied to Sephadex G-25 (GE Healthcare, UK) desalting columns with a molecular weight cut-off being 5 kDa to obtain eluates of high protein concentration and of low K^+ ion concentration (Almadaly *et al.*, 2015). Likewise, the collected eluates (desalted SP) were examined for Na^+, K^+ and Cl^- ions concentration using the automated equipment. Subsamples were dispensed from desalted SP for protein (60 µL) and lipid (100 µL) analyses. All subsamples of SP dispensed for protein, lipid and electrolytes analyses were kept in a −80 °C freezer until analysis.

Polyacrylamide gel electrophoresis (SDS-PAGE) of SPPs

The protein patterns of raw and desalted SP were assessed by SDS-PAGE accord-

ing to the method of Laemmli (1970). Subsamples of frozen raw and desalted SP were thawed at room temperature and diluted with an equal volume of electrophoresis sample buffer. The mixtures were incubated in boiling water bath for exactly 5 min. After centrifugation at 10000 × g for 5 min at 4 °C, the supernatants containing the proteins were collected and stored at −30 °C until electrophoresis. Extracted proteins were electrophoresed on 15% (w/v) polyacrylamide gel containing 0.1% SDS. The loading volume was 2 µL per lane in one gel and 5 µL per lane in another gel in trial for better detection of low and high molecular weight proteins, respectively. Electrophoresis was carried out in two steps; the first step was done at 15 mA per gel for exactly 1 h followed by 30 mA per gel at 25 °C until the dye front reached the lower end of the gel. The gel was immersed for a few minutes in a freshly prepared pre-fixative solution of 20% (v/v) methanol, 7.5% (v/v) acetic acid in milli-Q water. Protein bands were visualised with 0.1% Coomassie brilliant blue R-250 (Sigma-Aldrich, USA) staining at room temperature with gentle shaking for exactly 1 h (Bürk *et al.*, 1983); then destained in a freshly prepared solution of 30% (v/v) methanol, 10% (v/v) acetic acid in milli-Q water overnight with gentle shaking. Finally, the gels were scanned with a scanner (Epson PM-A900, Japan) and the apparent molecular weight was estimated using prestained protein marker, Broad range (7–175 kDa, New England, BioLabs, UK). The protein profile for each bull SP samples was run at least five times.

TLC of bovine SP lipids

Lipid extraction. Total lipids of both raw and desalted SP were extracted according to Bligh & Dyer (1959). Briefly, deep frozen (−80 °C) samples of raw and desalted SP were allowed to thaw at room temperature, 100 µL of each sample was diluted with 150 µL milli-Q water and kept in crushed ice. Chilled chloroform/methanol 1:2 (0.94 mL) was added to the diluted SP and vortexed vigorously for 5–15 s. Exactly, 310 µL chloroform and 310 µL milli-Q water were added and vortexed vigorously for exactly 30 s. After centrifugation at 1350 × g at room temperature for exactly 5 min, the lower phase containing the lipid extracts was dried under a stream of nitrogen gas in water bath at 37 °C. The obtained dry powder was kept in crushed ice until lipid separation.

Lipid separation. The separation of the neutral lipids was performed on silica gel $60F_{254}$ TLC plates using a solvent system of toluene–diethyl ether–ethanol–conc. NH_3 (250:200:10:1, v/v) according to Bocckino *et al.* (1987), whereas phospholipids were separated on HPTLC silica gel 60 plates using a solvent system of methyl acetate–isopropanol–chloroform–methanol–0.25% aqueous KCl (25:25:25:10:9, v/v) in agreement with Vitiello & Zanetta (1978). Exactly, 4 µg of 2-oleoylglycerol, cholesterol, DOG, 1,2-DMG and 1,3-DMG were spotted onto TLC plates on a separate lane as reference standards of neutral lipids. Similarly, 10 µg of L,α-PC, L,α-PE, L,α-PS, L,α-PI, SM and Lyso-PC were spotted into HPTLC plates on a separate lane as reference standards of phospholipids. These reference standards were included in all plates due to differences in separation among plates. Chromatography was performed at room temperature until the developing solvents migrated to the top edge of the HPTLC plates for phospholipids and to 3 cm below the top edge of TLC plates for neutral lipids.

Detection of lipid spots. Neutral lipid spots on TLC plates were stained with 0.03% Coomassie brilliant blue R-250 in 30% methanol/100 mM NaCl in milli-Q water at room temperature for 30 min (Nakamura & Handa, 1984); after well-draining of plate, it was destained by a freshly prepared solution of 30% (v/v) methanol/100 mM NaCl in milli-Q water for 5 min with gentle shaking. After de-staining the plate was drained well and kept at room temperature in dark place overnight to dry. Finally, the TLC plate was scanned with a scanner (Epson PM-A900, Japan).

The identities of phospholipid spots on HPTLC were visualised after charring the plate with freshly prepared 10% sulfuric acid (H_2SO_4) and heated in an oven (Yamato-Japan) at 110 °C for 30–60 min (Kritchevsky *et al.*, 1973). Similarly, HPTLC plate was scanned with the same scanner.

RESULTS

Seminal plasma desalting

The concentration of Na^+, K^+ and Cl^- ions in raw and desalted SP of Holstein-Friesian bulls is shown in Table 1. The obtained results revealed that Na^+ ion concentration in raw and desalted SP was identical (< 75 meq/L) in all bulls except those of No. 1, 2 and 6 as well as pooled SP it was 100, 97, 104 and 78 meq/L respectively in raw SP, which decreased to <75 meq/L after gel filtration. Noteworthy, K^+ ion concentration in raw SP was ≥12.1 meq/L but after gel filtration it was <1 meq/L in all bulls except those of No 4 and 10 as well as pooled desalted SP it was 1.8, 1.6 and 1.6 respectively. On contrary, Cl^- ions concentration was not affected with gel filtration in all bulls except bull No. 6 it decreased from 79 meq/L to < 50 meq/L (Table 1).

Table 1. Na^+, K^+ and Cl^- ions concentrations of raw and desalted SP from Holstein-Friesian bulls

Bull	Electrolytes profile (meq/L)*					
	Na^+		K^+		Cl^-	
	Raw SP	Desalted SP	Raw SP	Desalted SP	Raw SP	Desalted SP
B1	100	< 75	> 14	< 1	< 50	< 50
B2	97	< 75	12.1	< 1	< 50	< 50
B3	< 75	< 75	> 14	< 1	< 50	< 50
B4	< 75	< 75	> 14	1.8	< 50	< 50
B5	< 75	< 75	> 14	< 1	< 50	< 50
B6	104	< 75	> 14	< 1	79	< 50
B7	< 75	< 75	> 14	< 1	< 50	< 50
B8	< 75	< 75	> 14	< 1	< 50	< 50
B9	< 75	< 75	> 14	< 1	< 50	< 50
B10	< 75	< 75	> 14	1.6	< 50	< 50
Pooled SP (B1~10)	78	< 75	> 14	1.6	< 50	< 50

*Na^+, K^+ and Cl^- concentrations in raw and desalted SP were determined in order to evaluate the efficacy of Sephadex-G desalting column.

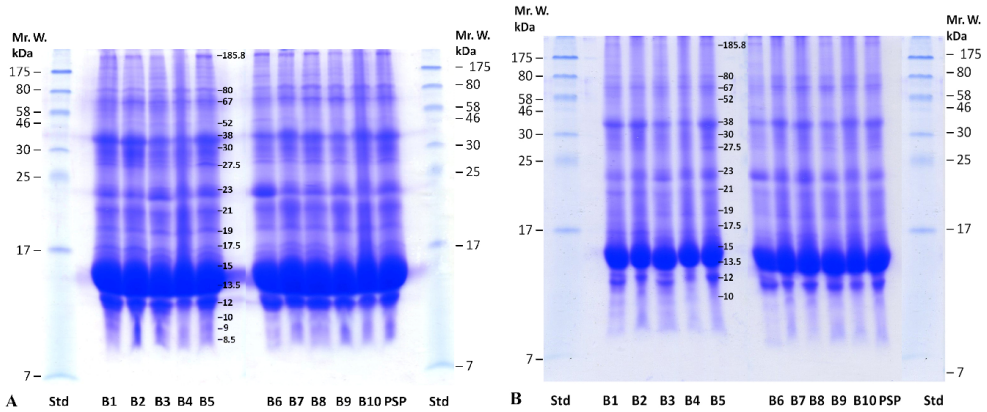

Fig. 1. SDS-PAGE of bovine raw SP by loading 5 µL **(A)** and 2 µL **(B)** per lane. SSPs were reduced, denatured and separated on 15% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Std=standard; B=bull; PSP=pooled SP; Mr.W=molecular weight; kDa = kilodalton.

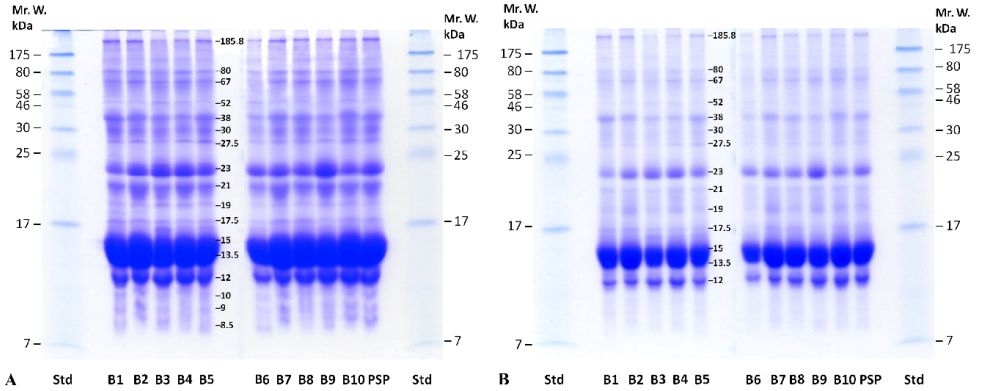

Fig. 2. SDS-PAGE of bovine desalted SP by loading 5 µL **(A)** and 2 µL **(B)** per lane. SSPs were reduced, denatured and separated on 15% polyacrylamide gel and stained with Coomassie brilliant blue R-250. Std=standard; B=bull; PSP=pooled SP; Mr.W=molecular weight; kDa = kilodalton.

Protein analysis

Although the concentration of TP in SP was variable among bulls it ranged from 7.0 to 10.4 g/dL in all bulls except bull No. 6 has a relatively low TP concentration of 4.9 and 6.8 g/dL for its first and second ejaculates respectively. On the other hand, bull No. 4 had the highest TP concentration either in the first (10.2 g/dL) or the second (10.4 g/dL) ejaculates. Also, the overall mean of TP concentration in the first ejaculate (8.02 ± 0.44) was insignificantly different from that of the second ejaculate (8.16 ± 0.35). Interestingly, SDS-PAGE of raw (Fig. 1A,B) and desalted (Fig. 2A,B) SP revealed no substantial difference in the detected SPPs. Only protein bands in desalted SP were sharp and well demarcated than those of raw SP especially those with low molecular masses such as of 13.5 and

15 kDa which appeared as a doublet on the gel (Fig. 2B). In total, 17 protein bands of 185.8, 80, 67, 52, 38, 30, 27.5, 23, 21, 19, 17.5, 15, 13.5, 12, 10, 9 and 8.5 kDa were detected. Regarding the protein profile of each bull our data revealed that there were no clear differences among bulls except those of No. 1, 2, 5, 7, 8 and 10 having much SPPs compared with other bulls particularly bull No. 6 depending upon the size and the staining intensity of protein bands.

Expectedly, by increasing the loading volume of sample from 2 µL to 5 µL the protein bands of low concentration such as those of 80, 67, 52, 30, 27.5, 21, 19 and 17.5 kDa became obvious and strongly stained as shown in Fig. 1A and 2A. However, for good separation of low molecular weight proteins 2 µL (Fig. 1B and 2B) was better than 5 µL (Fig. 1A and 2A). For instance, protein bands of 13.5 and 15 kDa appeared as distinct bands specifically in desalted SP (Fig. 2B), but when higher (5 µL) volume of raw SP was loaded it appeared as smear or diffuse bands (Fig. 1A). Moreover, Fig. 1 and 2 showed that bands of 38, 23, 15, 13.5 and 12 kDa represented the major bovine SPPs according to the band size and its staining intensity. Noteworthy, a high molecular weight protein of 185.8 kDa was detectable in raw, desalted and pooled SP of all Holstein-Friesian bulls.

Lipid analysis

Neutral lipids. Fig. 3 shows the neutral lipids profile of raw (Fig. 3A) and desalted (Fig. 3B) SP. Neutral lipids profile of raw SP appeared to be similar to that of desalted SP except the staining intensity of lipid spots which was darker, denser and well demarcated in raw SP than that of desalted SP. The detected neutral lipid spots were cholesterol and ones corresponding to 1,2-DMG, 1,2-DOG, 1,3-DMG and 1,3-DOG whereas, phospholipids did not migrate on TLC plate and remain on the site of samples spotting as shown in Fig. 3A and B. Depending on the size and the staining intensity of the spots cholesterol seems to be the main component of neutral lipids in Holstein-Friesian bull's SP and there was no clear

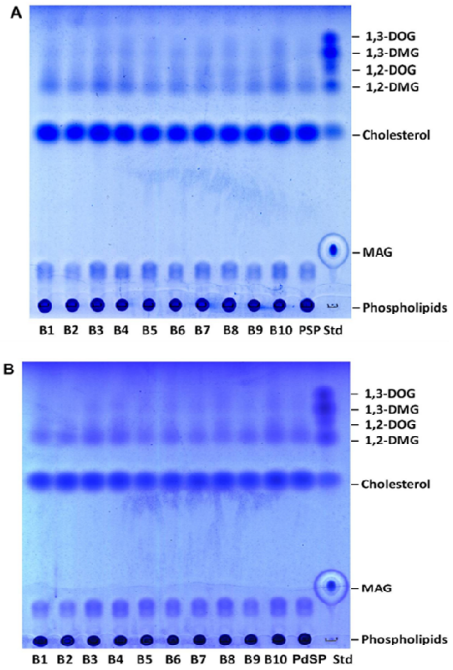

Fig. 3. Neutral lipids profile of raw **(A)** and desalted **(B)** SP of Holstein-Friesian bulls. Silica gel $60F_{254}$ TLC plates developed in toluene–diethyl ether–ethanol–conc. NH_3 (250:200:10:1, v/v). Neutral lipid spots on TLC plates were stained with 0.03% Coomassie brilliant blue R-250. All lanes were loaded with its corresponding bull (B) number (1–10). Std=standards of neutral lipids (cholesterol, 2-oleoylglycerol, DOG, 1,2-DMG and 1,3-DMG; 4 µg each); B=bull; PSP=pooled SP; MAG=monoacylglycerol; 1,2-DMG=1,2-dimyristoyl-sn-Glycerol; 1,2-DOG=1,2-dioleoglycerol; 1,3-DMG=1,3- dimyristoyl-sn-Glycerol; 1,3-DOG=1,3-dioleoglycerol.

difference in the detected neutral lipids among bulls. Surprisingly, unknown neutral lipid spot was detected in both raw and desalted SP of all bulls which was expected to be monoacylglycerol (MAG)

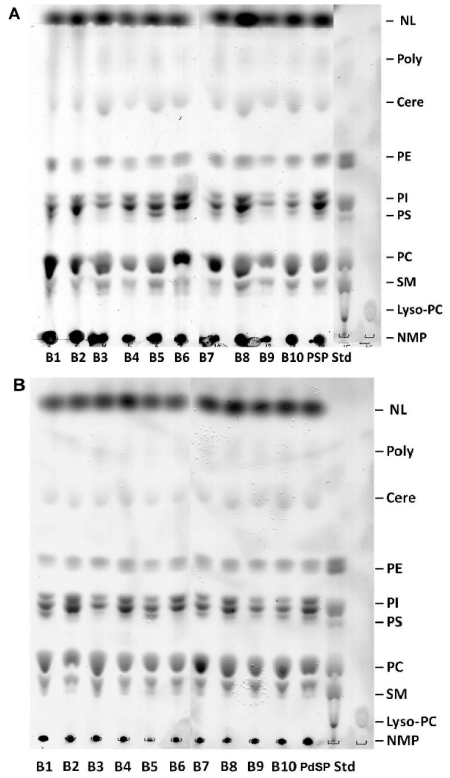

Fig. 4. Phospholipids profile of raw **(A)** and desalted **(B)** SP of Holstein-Friesian bulls. Silica gel 60 HPTLC plates developed in methyl acetate–isopropanol–chloroform–methanol–0.25% aqueous KCl (25:25:25:10:9, v/v). Phospholipids spots on HPTLC plates were detected after charring the plate with freshly prepared 10% sulfuric acid (H_2SO_4) and heating at 110 °C for 30–60 min. Std=standard phospholipids (Lyso-PC, SM, PC, PS, PI and PE; 10 µg each); B=bull; PSP=pooled SP; NMP=non-migrating phospholipids; Lyso-PC=lyso-phosphatidylcholine; SM=sphingomyelin; PC=phosphatidylcholine; PS=phosphatidylserine; PI=phosphatidylinositol; PE=phosphatidylethanolamine; Cere=cerebroside; Poly=polyglycerol phosphatide; NL=neutral lipids.

but unfortunately, it appeared on a lower level than MAG standard on the TLC plate as shown in Fig. 3A and B.

Phospholipids. Even though the detected phospholipid spots in raw (Fig. 4A) and desalted (Fig. 4B) SP were identical, but raw SP containing denser and darker spots especially non-migrating phospholipids (NMP), PC and PI. The detected phospholipids were: NMP, SM, PC, PS, PI, PE, cerebroside (Cere) and polyglycerol phosphatide (Poly), but Lyso-PC could not be detected on HPTLC plates as shown in Fig. 4A and B. Notably, SM, PC, PI and PE represented the major phospholipids in raw and desalted SP of Holstein-Friesian bulls. Moreover, PI was variable among bulls where it was detected as two spots in bulls No. 2, 3, 6 and 9 but, it appeared as three spots in those of No. 1, 4, 5, 7, 8 and 10 especially in desalted SP (Fig. 4B).

DISCUSSION

The present study represents a continuing effort following primary work focused on the incorporation of desalted and lyophilised SP into cryoprotective diluent before cryopreservation to minimise the freeze-thaw damage of bull spermatozoa (Almadaly *et al.*, 2015). Semen quality is considered a risk factor for subfertility and/or infertility in dairy and beef cattle farms. Thus, the conventional semen characteristics such as volume, motility and concentration used to evaluate the potential fertility of semen sample account for the differences in the fertility of bulls (Sharma *et al.*, 2012). Moreover, the collected ejaculate should have a uniform near-white appearance. Semen from some bulls contain greater amounts of riboflavin, which results in a yellowish-coloured semen (White & Lincoln, 1960), but defi-

nitely these ejaculates will not have a urine smell. However, in the current study, no remarkable relationship was noticed among semen characteristics, TP concentration and/or the identified protein or lipid species in bovine SP, but bull No. 6 had low proportion of progressive motility and low sperm concentration which was consistent with the lower TP concentration of its SP compared with other bulls. Also, this might be attributed to the higher concentration of Na^+, K^+ and Cl^- ions in raw SP of this bull (B6) compared with other bulls.

SP is a physiological secretion from the accessory glands of male genital system, which plays an important role in the maturation of the spermatozoa through hormonal, enzymatic and surface-modifying events (Juyena & Stelletta, 2012). Since it is known that novel and unidentified roles of some proteins exist within each bull species, an attempt have been carried out to characterise the proteome map of SP belonging to fertile bulls. Here, analysis of protein components in Holstein-Friesian bull's SP revealed that, a total of 17 SPPs of 185.8, 80, 67, 52, 38, 30, 27.5, 23, 21, 19, 17.5, 15, 13.5, 12, 10, 9 and 8.5 kDa were detected as shown in Fig. 1 and 2. Protein band of 67 kDa may correspond to albumin or clusterin precursor or to ecto-5′-nucleotidase and that of 52 kDa may correspond to osteopontin (OPN) as summarised in Table 2 (Moura *et al.*, 2006, 2007). Protein bands of 38, 23, 13.5 and 12 kDa may correspond to cathepsin B or D, tissue inhibitor metalloproteinase-2 (TIMP-2), spermadhesin Z13 and acidic seminal fluid protein (aSFP), respectively as presented in Table 2 according to Moura *et al.* (2006, 2007).

Protein bands of 30, 15 and 13.5 kDa may correspond to BSP-30 kDa, BSP-A1 or BSP-A3 and BSP-A2, respectively, according to Desnoyers *et al.* (1994). This

Table 2. Protein bands identified in this study with reference to known proteins

Estimated molecular weight (kDa)	Candidate protein (molecular weight, kDa)
185.8	Novel protein or might be fast myosin heavy chain extraocular (Odhiambo & Dailey 2011)
80	Novel protein
67	Albumin (71) or clusterin precursor (70) or ecto-5′-nucleotidase (63)
52	OPN (55) or clusterin (51.1) or PLA_2 (50.1) or platelet-activating factor acetylhydrolase (53.2).
38	Cathepsin B (36.7) or cathepsin D (42.5)
30	BSP-30 kDa (28–30)
27.5	Novel protein
23	TIMP-2 (25–26)
21	Novel protein
19	Novel protein
17.5	Novel protein
15	BSP-A1 (15) or BSP-A3 (16.5)
13.5	BSP-A2 (15–16) or spermadhesin Z13 (14)
12	aSFP (12–14)
10	Novel protein or secretoglobin 1D (Boe-Hansen *et al*., 2015)
9	Novel protein
8.5	Novel protein

would explain the fact that these two proteins (15 and 13.5 kDa) migrated as doublet on polyacrylamide gel as shown in Fig. 1B and 2B in agreement with Manjunath *et al.* (1987). The main point of interest is that protein bands of molecular masses 80, 27.5, 21, 19, 17.5, 9 and 8.5 kDa may be candidates of bovine SPPs that need further investigations. Both the current study and our recent study (Almadaly *et al.*, 2015) are unique in detection of high (185.8 kDa) molecular weight protein which may be a novel SPP in bovine semen or it may correspond to fast myosin heavy chain extraocular according to Odhiambo & Dailey (2011). Also, the detected protein of 10 kDa may correspond to secretoglobin 1D according to Boe-Hansen *et al*. (2015). The protein and lipid species of both raw and desalted SP were identical; this emphasises the efficiency of our desalting technique because it did not affect protein and/or lipid species of SP but affect on Na^+, K^+ and Cl^- ions concentration. Thus, desalted SP containing a lower concentration of electrolytes when incorporated into semen diluents prior to cryopreservation may avoid the detrimental effect of these electrolytes especially bicarbonate and K^+ as well as may protect sperm cell from cryocapacitation in accordance with Almadaly *et al.* (2015). These results provide justification for further studies to explore and better understand the mechanisms by which SPPs influence bull fertility.

Since higher concentration of SPPs was present in 5 µL loading volume than in 2 µL, consequently, more intense and distinct bands were detectable on polyacrylamide gels loaded with 5 µL especially protein bands of 13.5 and 15 kDa. Moreover, 5 µL loading volume was better than 2 µL to detect the low abundance SPPs such as those of 80, 67, 52, 30, 27.5, 21, 19, 17.5, 10, 9 and 8.5 kDa. However, four SPPs [OPN, spermadhesin Z13, BSP-30 kDa and phospholipase A_2 (PLA_2)] have been identified as a fertility biomarkers in dairy bulls (Moura *et al.*, 2006, 2007), but in the current study there are new candidates of fertility-associated proteins at least in Holstein-Friesian bull's SP such as those of 38, 23, 21, 15, 13.5 and 12 kDa which need comprehensive proteomic studies using animals with varying fertility.

The major bovine SPPs are BSP-A1/A2, BSP-A3 and BSP-30 kDa which play substantial roles in fertility by maintaining sperm in an appropriate state in the female genital tract until the oocyte reaches the site of fertilisation (Yu *et al.*, 2003; Gwathmey *et al.*, 2006). Our results are partially consistent with these findings because protein bands of 15 and 13.5 kDa which might correspond to BSP-A1 or BSP-A3 and BSP-A2, respectively were the most abundant proteins in these fertile bulls. In addition, protein bands of 12, 23, and 38 might be new candidates of fertility-associated proteins in bovine SP because these bands were predominant at least in fertile bulls used in the current study. Our results support the findings of Killian (2012) who identified putative fertility-associated proteins like BSP-30 kDa, prostaglandin-D synthase, OPN and PLA_2. It is inferred that electrophoresis of Holstein-Friesian bull SPPs provided some new information when compared with previous studies namely: 1) our study was unique to detect protein of 185.8 kDa in bovine SP; 2) higher loading volume was recommended for detection of minor SPPs whereas, lower loading volume was recommended for better separation of major SPPs and 3) there was breed difference in some SPPs such as of 58.6, 41.8, 34, 32, 30, 27.5, 25.2, 23, 9, 8.5, 6.7 and

5.2 kDa between Japanese Black bulls (Almadaly *et al.*, 2015) and Holstein-Friesian bulls used in the current study.

Sperm cell structure and function depend on the lipid composition in sperm cells and SP (Barranco *et al.*, 2015; Wood *et al.*, 2016) which in turn affect its fertilisation capacity. Cholesterol is a key factor in semen quality and function where analysis of semen samples from stallions with unexplained subfertility revealed a low concentration of SP cholesterol (Brinsko *et al.*, 2007). Also, low cholesterol concentration was demonstrated in the known poor semen samples of bulls taken during summer, even when evaluated as good on the basis of sperm count and morphology (Argov-Argaman *et al.*, 2007). Along with phospholipids, cholesterol is necessary for cell physical integrity because cholesterol efflux from the sperm plasma membrane initiates the key step in sperm capacitation and acrosome reaction that is crucial for fertilisation (Witte & Schäfer-Somi, 2007). Moreover, adding cholesterol to diluents prior to freezing reduces freezing-thawing damage, preserving sperm motility and fertilisation potential (Moore *et al.*, 2005) and reduces premature capacitation during cryopreservation of bull spermatozoa (Almadaly *et al.*, 2015).

Neutral lipid species of raw and desalted SP were similar wherein, the same spots of neutral lipid were detected. This result was parallel to the above-mentioned protein analysis which means that our desalting technique was successful because it did not affect protein and/or lipid species of SP. The spots corresponding to 1,2-DMG and 1,2-DOG have been identified as 1,2-diacylglycerol (DAG) with 2 saturated fatty acid chains (designated as disaturated diacylglycerol; 1,2-DS-DAG), 1,2-diacylglycerol with one unsaturated and the other saturated fatty acid chains (saturated, unsaturated diacylglycerol; 1,2-SU-DAG) (Roldan & Murase, 1994). This finding is consistent with our earlier observation (Almadaly *et al.*, 2015) in Japanese Black bull SP. To our knowledge both the current and our recent (Almadaly *et al.*, 2015) reports were unique to have detected 1,2-DS-DAG, 1,2-SU-DAG and DAGs corresponding to 1,3-DMG and 1,3-DOG diacylglycerol species respectively, in bovine SP. Argov-Argaman *et al.* (2013) reported the presence of high amounts of myristic, palmitic, oleic, linoleic, adrenic and docosahexaenoic fatty acids in bovine SP. In the light of this finding the unknown neutral lipid spot detected herein (below MAG), might be one of these fatty acids or it might correspond to 1-MAG (saturated or unsaturated form) or to 2-MAG (saturated or unsaturated form) which warranted further lipidomic analysis in our laboratory.

Phospholipids are primary components of membrane which are essential to sperm-oocyte fusion, emphasising our interest in its analytical methodology. Lipid analysis by TLC will further increase because TLC is even nowadays considered to be the method of choice if large numbers of samples have to be routinely screened. However, HPTLC plates offer the best resolution but TLC plates are convenient for loading larger amounts of sample (Schnaar & Needham, 1994). Surprisingly, TLC of bovine SP indicated the presence of two phospholipid constituents migrating near the solvent front. These two constituents were identified as polyglycerol phosphatide (Poly) and cerebroside (Cere) according to Pursel & Graham (1967). Poly was the smallest phospholipid fraction in bull SP which also has previously been reported to be a constitu-

ent of ram spermatozoa by Hartree & Mann (1961). Phospholipid remaining at the spotting area was labelled NMP and was quite variable, representing 4 to 7% of the total phospholipids. NMP was probably composed of a little of each of the phospholipid fraction since it gave a positive reaction for choline and amino groups (Johnson *et al.*, 1969). Unfortunately, Lyso-PC was undetectable which might be due to its scarce amount in SP in agreement with Johnson *et al.* (1969). Our data revealed that the phospholipids profile of SP before and after gel filtration was similar and the order in which phospholipids appeared on HPTLC was: NMP, SM, PC, PS, PI, PE, Cere and Poly in agreement with Pursel & Graham (1967) using the same solvent system of methyl acetate-isopropanol-chloroform-methanol-0.25% aqueous KCl (25:25:25:10:9, v/v) and with Johnson *et al.* (1969), who used chloroform–methanol–acetic acid–H_2O (25:15:4:2 v/v) solvent system.

However, PI occurs only in very small amounts in SP and it is much more difficult to extract (Singh & Jiang, 1995; Wood *et al.*, 2016). Herein, PI appeared as three separate spots in agreement with Almadaly *et al.* (2015) which might correspond to PI-monophosphate (PIP); PI-biphosphate (PIP_2) and PI-triphosphate (PIP_3) because PI can be further phosphorylated on the inositol ring (Fuchs *et al.*, 2011). The predominant phospholipids fraction within both spermatozoa and SP were PC and PE (Kelso *et al.*, 1997) in accordance with our results, PC and PE were within the major components of SP phospholipids in fertile Holstein-Friesian bulls depending on spot size and its staining intensity.

In conclusion, there was no difference in the protein and lipid species between raw and desalted SP. Cholesterol represents the major neutral lipid whereas SM, PC, PI and PE represent the major phospholipids in bovine SP. TLC remains the technique of choice in separation of the various lipid species. One major progress was achieved when HPTLC was introduced that enabled lipid separation with much higher quality and sensitivity especially with the PI subspecies. Although, this study gave a general picture of protein and lipid species in fertile bull SP, further investigation of the identified protein and lipid species in large samples are essential to ascertain their applicability as biomarkers of bull fertility.

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