

## Protein profile and functionality of spermatozoa from two semen collection methods in Bali bulls



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### ARTICLE INFO

#### Article history:

Received 23 June 2014

Received in revised form

2 December 2014

Accepted 8 December 2014

#### Keywords:

Bali bull

Electro-ejaculation

Transrectal massage

Semen protein

### ABSTRACT

The aim of this study was to evaluate the effect of two semen collection methods (electro-ejaculation [EE] and transrectal massage [RM]) on in vitro sperm functionality and protein composition of seminal plasma in Bali bulls. Ten untrained Bali bulls were selected for semen collection by EE and RM. Parameters analysed were acrosome and plasma membrane integrity, sperm motility (by CASA), normal morphology, functionality (sperm penetration assay), acrosome reaction, total protein content and protein profiles (by 2D-PAGE). Bulls collected by RM had a higher ( $p < 0.05$ ) percentage of spermatozoa with intact acrosome and plasma membrane, functionality and individual motility, and a lower proportion of seminal plasma, total protein content and lower ratio of low molecular weight proteins than those collected by EE. Analysis of 2D-PAGE gel detected about 116 spots in the range of 10–250 kDa and isoelectric points (pI) ranging from 3 to 10. Approximately 52% of seminal plasma protein spots were represented by four major protein fractions with molecular weights around 37–45 kDa (15.66%), 25–30 kDa (12.46%), 14–16 kDa (11.73%) and 12–15 kDa (11.52%). Ten of the seminal plasma proteins identified by mass spectrometry belonged to major bovine seminal plasma proteins. A very significant finding in this study was related to the two proteins identified, PGK and PLA<sub>2</sub>, with MW of approximately 37–40 and 50–55 kDa and pI of 8.5–8.8 and 5.2–6.0, respectively. These two protein spots can only be detected in the seminal plasma of ejaculates obtained through RM. In conclusion, semen quality as examined by in vitro sperm functionality was found to be better in RM than EE samples after treatment with heparin and calcium ionophore A-23187. In addition some low molecular weight proteins were up-regulated in the seminal plasma obtained from the EE method.

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### 1. Introduction

Bali cattle (*Bos javanicus*) are popularly raised in Indonesia and have been introduced into Malaysia. Semen

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characteristics of Bali bulls have not been widely studied since the bulls have not been trained for semen collection. Semen samples are commonly collected from live bulls either by an artificial vagina (AV) as conducted by Barszcz et al. (2012) and Barth et al. (2004) or via an electro-ejaculator (EE) as reported by Andrews (1967), Ball (1973), Dziuk et al. (1954a), Palmer et al. (2004) and (2005). Nevertheless semen collection via AV is the preferred method of collection over EE for domestic bulls because it resembles natural breeding (Santiago-Moreno et al., 2009; Wulster-Radcliffe et al., 2001). This technique however requires a trained bull (Wulster-Radcliffe et al., 2001) and occasionally this method can become quite difficult and time consuming if the bull is not cooperative or becomes irritable (Palmer et al., 2013). On the other hand, EE which is usually used in untrained bulls can be performed repeatedly with a large number of bulls per session, without causing death to the bulls (Santiago-Moreno et al., 2009). Another semen collection method which has been described in cattle (Palmer et al., 2004; 2005) is the transrectal massage (RM). Transrectal massage when directed specifically on the ampullary glands located at the dorsal neck of the urinary bladder has been proven to be quite effective in obtaining semen samples from bulls (Palmer et al., 2004; Sarsaifi et al., 2013a).

The seminal plasma serves as a vehicle for ejaculated sperm and is a composite medium of a great variety of molecules produced by the accessory sex glands (Boisvert et al., 2004; Mann, 1954; Mann and Lutwak-Mann, 1976; Manjunath and Thérien, 2002) that are mixed with sperm during ejaculation. It contributes to the main portion of semen in terms of volume and composition (Moura et al., 2006a). It is well known that seminal plasma comprises different components (proteins, enzymes, lipids, organic acids, minerals) and each component has an important role in sperm function (Chacur, 2012). Studies have shown that factors in the seminal plasma (such as proteins) can both positively and negatively affect sperm fertility parameters (Killian et al., 1993; Kumar et al., 2009; Miller et al., 1990), such as viability (Dott et al., 1979) and motility (Iwamoto and Cagnon, 1988). In an analysis of seminal plasma from Holstein bulls of varying fertility, four proteins were found involved in bull fertility, and two of these four seminal plasma proteins were reported to be positively correlated with fertility (Killian et al., 1993).

Some proteins in the bovine seminal plasma (BSP) are adsorbed onto the sperm surface during ejaculation (Desnoyers et al., 1994; Manjunath et al., 2002) to maintain membrane stability before capacitation occurs in the female genital tract (Manjunath et al., 2002). The preconditions for fertilization to occur are capacitation and acrosome reaction of the spermatozoa (Nixon et al., 2007). Capacitation is enhanced by heparin binding proteins (Miller et al., 1990). The protein composition of mammalian seminal plasma varies among species (Boisvert et al., 2004; Brandon et al., 1999; Cross, 1993; Frazer et al., 1996; Mortarino et al., 1998; Muiño-Blanco et al., 2008; Villemure et al., 2003), season (Domínguez et al., 2008; Giuliano et al., 2008) and the method of seminal collection (Giuliano et al., 2008; Marco-Jiménez et al., 2005). Thus, the exact nature of the protein components is not well-known. A study was conducted with the objective of evaluating the effects of two

semen collection methods (RM or EE) on sperm quality of frozen-thawed Bali bull semen after treatment with heparin and calcium ionophore A-23187. In addition, the protein content and protein profile were compared between the two different methods of semen collection.

## 2. Materials and methods

### 2.1. Animals and management

Ten 2 to 4 year-old Bali bulls with body weights ranging from 150 to 220 kg were used in this study. The bulls were raised in oil palm plantations at the FELDA farms in Negeri Sembilan, Malaysia (2°45'0" North, 102°15'0" East) at an altitude of about 27 m above sea level, with an annual rainfall of 22.49 cm. The ambient temperature and relative humidity during the period of study ranged from 22 to 34 °C and 65 to 85%, respectively. The animals were allowed to graze the vegetative undergrowth comprising native grasses and broad-leaf weeds found in the oil palm plantations and given feed supplement of palm kernel cake at a rate of 1.5 kg per animal per day, and water was provided ad libitum.

### 2.2. Semen collection

The ten untrained mature and healthy Bali bulls were subjected to EE and RM methods in this study. There were no records on the breeding activities of the bulls before the study. Breeding soundness examination (BSE) was performed before semen collection and all ten bulls were in satisfactory condition. The electro-ejaculator (ElectroJac5 Ideal® Instruments, Neogen Corporation, Lansing, MI, USA) was equipped with 6.5 cm diameter transrectal probe with three ventrally oriented electrodes. The electrical stimulation was gradually increased until the bull ejaculated. An initial stimulus of 1 V was applied to the bull with subsequent stimulations gradually increased (maximum of 10 V) depending on the bull's ejaculation response towards EE. Every stimulus lasted for 8–10 s and then paused for about 2 s before the next stimulus was given. For the RM method, transrectal massaging was performed by back and forth hand motions over the region of the ampulla, prostate and seminal vesicles. This collection technique followed that of Palmer et al. (2004) and Sarsaifi et al. (2013b), until semen was discharged. The average time for transrectal massage lasted 2 min per bull. For each method of collection, two semen samples were collected per bull at a sampling interval of 3–4 days. Each semen sample comprised two tubes of ejaculates that were pooled. Semen samples containing foreign materials such as blood, bedding, dust, urine, fecal materials and pus were discarded before pooling. The interval between the two collection methods was 1–2 weeks. In the semen collection procedure, four to five trained personnel were involved, and each personnel were tasked with a specific part of the procedure. This was to avoid individual effect for the two collection methods. In total 40 semen samples were collected from the ten bulls on two sampling days using the two collection methods.

### 2.3. Semen evaluation

Immediately after collection all of the semen samples were evaluated on the farm for volume, colour, pH, concentration and motility following the guidelines of the Western Canadian Association of Bovine Practitioners (Barth, 2000). Sperm concentration was determined using an Accucell photometer (IMV Technologies, L'Aigle, France), whilst pH of the semen was determined using a mobile pH meter (CT-6021A, Mettler-Toledo LLC, Columbus, OH, USA). From each pooled semen sample, one ml was taken for the collection of seminal plasma and the remaining was processed for cryopreservation.

### 2.4. Semen cryopreservation

Semen samples from each sampling day were extended with BIOXcell (IMV Technologies, L'Aigle, France) at 37 °C and equilibrated at 4 °C for 3 h before placed into 0.5 ml straws. Filled straws were arranged on a special rack, 4 cm above liquid nitrogen and exposed to liquid nitrogen vapour for 9 min, and then plunged into a cryogenic tank filled with liquid nitrogen (−196 °C) until in vitro sperm functionality analysis.

### 2.5. Seminal plasma collection

The semen samples were centrifuged at 10,000 × g for 15 min at 4 °C. After centrifugation, the supernatant containing the seminal plasma was aspirated into 10 ml Eppendorf tubes and preserved by adding 10% protease inhibitor cocktail, then divided into aliquots and stored at −80 °C until analysis.

### 2.6. in vitro sperm functionality

Twenty straws (two straws from each bull) containing about 25–30 million live sperm per straw from each collection method were used for the in vitro experiments on sperm functionality that were conducted in three replicates. The straws were thawed and pooled across bulls to avoid individual bull effects.

### 2.7. Sperm capacitation with heparin

Frozen semen straws were thawed in a water bath (37 °C) for 30 to 45 s, pooled and mixed by pipetting in a 1.5 ml conical tube (Fisher Scientific, Inc., Pittsburgh, PA). Swim-up method was used for sperm separation by Sperm-TALP medium (Parrish et al., 1988) supplemented with fatty acid-free bovine serum albumin (6 mg/ml) and sodium pyruvate (1 mM/ml). The final pellet obtained after swim up was diluted with 100 µg/ml heparin (Sigma No. H-5765) to obtain a final sperm concentration of 2–4 million sperm/ml of Sperm-TALP medium. Sperm suspension was further incubated in a CO<sub>2</sub> incubator for 15 min at 38.5 °C to allow them to undergo sperm capacitation. The capacitation status was assessed under a light microscope at 400 × magnification by penetration of spermatozoa into the oocyte. in vitro sperm functionality was determined by the following procedures:

#### (1) **Acrosome reaction (AR)**

To induce the acrosome reaction, 10 µM calcium ionophore A-23187 was added to Sperm-TALP medium in the last 10 min of incubation (Tardif et al., 1999). Acrosome reaction was evaluated using a simplified triple stain technique as described by Talbot and Chacon (1981). At least 400 sperm were examined and the criteria used to identify dead and live sperm with and without acrosome reaction were based on descriptions by Ferrari and Barnabe (2000) and Talbot and Chacon (1981).

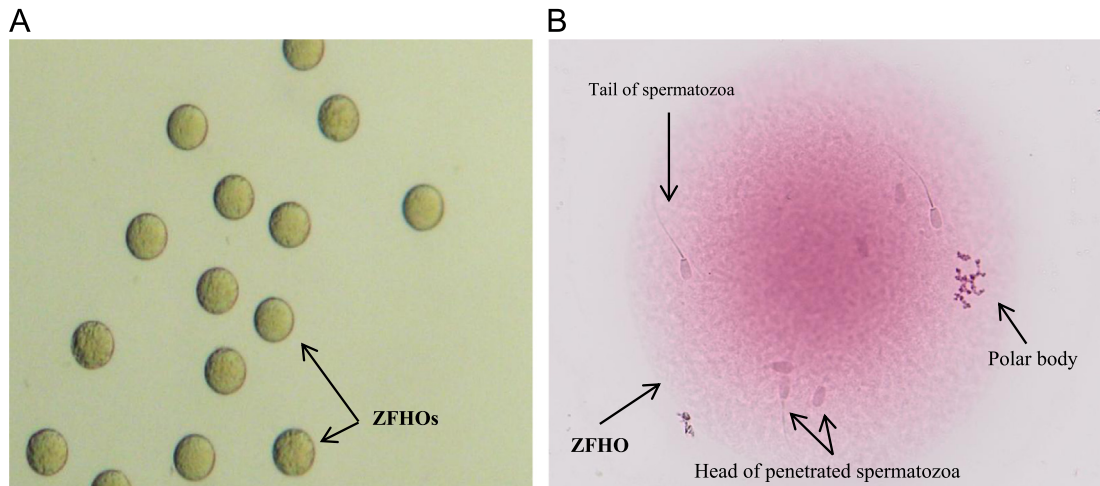
#### (2) **Sperm motility parameters by CASA**

Computer-assisted semen analysis with the CASA IVOS 10 system (Hamilton Thorne Bioscience, Beverly, MA, USA) was used to assess sperm motility on individual trajectories of spermatozoa motility (%), progressive motility (%), average path velocity (VAP, µm/s), straight line velocity (VSL, µm/s), curvilinear velocity (VCL, lm/s), amplitude of lateral head displacement (ALH, lm), beat cross frequency (BCF, Hz), straightness (STR, as VSL/VAP,%) and linearity (LIN, as VSL/VCL,%) of each frozen Bali semen sample collected by RM and EE methods. From each sample, three slides were prepared as replicates. The software was set based on the manufacturer's recommendation for the assessment of motility characteristics of bull spermatozoa. As recommended by the manufacturer, the setting of the equipment was set as follows: frames acquired: 30; frame rate: 60 Hz/s; straightness threshold: 70%; slow VAP cut off: 30 µm/s; progressive VAP: 50 µm/s; slow VSL cut off: 15 lm/s; minimum contrast for cell detection: 80; minimum cell size: 5 pixel; and magnification factor: 1.92. After each scan, the playback facility performed a quality control of the correct acquirement of the field. For each sample, four microscopic fields were analysed, and a minimum of 200 spermatozoa were evaluated.

#### (3) **Sperm penetration assay**

The sperm penetration assay in this experiment followed the procedure described previously for human spermatozoa (Yanagimachi et al., 1976). Modified Tyrone's solution (TALP; Bavister and Yanagimachi, 1977) was used for handling oocytes and spermatozoa. Fifty six mature golden Syrian hamster females (*Mesocricetus auratus*) were superovulated by intraperitoneal administration of 40 IU PMSG (Folligon® Intervet International, B.V. UK) and 56 h later with 45 IU of hCG (Chorulon® Intervet International, B.V. UK). Sixteen to 18 h later, the hamsters were euthanized and the oviducts recovered, cumulus mass was removed from the oocytes after adding 0.1% (w/v) hyaluronidase (Type I, H-3506; Sigma-Aldrich) in TALP medium for 2–3 min. The zona-pellucida was removed using 0.1% (w/v) trypsin Type III in TALP medium (T-8003; Sigma-Aldrich) according to the technique described by Sarsaifi et al. (2013).

The capacitated spermatozoa were re-suspended in Fert-TALP medium (Bavister and Yanagimachi, 1977) at the required concentration of 2–4 × 10<sup>6</sup> sperm/ml for 1 h at 37 °C. Zona-free hamster oocytes (ZFOs) were inoculated immediately with a drop of sperm (100 µl) and co-incubated at 37 °C for 3 h in a 5% CO<sub>2</sub> incubator.



**Fig. 1.** (A) Zona free hamster oocytes (ZFHOs); Frozen-thawed Bali bull spermatozoa after capacitation incubated with ZFHOs. (B) Polar body of ZFHO, head and tail of penetrated spermatozoa are indicated by arrows.

Between 40 and 60 oocytes were used for each semen sample. Successful penetration of an oocyte by the sperm was determined according to the criteria described by Yanagimachi et al. (1976). ZFHOs were washed in drops of TALP medium, fixed in 3:1 methanol: acetic acid overnight and stained with 1% aceto-orcein. Scoring was done as a fertilization percentage (FP) calculated as total number of oocytes penetrated divided by total number of oocytes used and multiplied by 100 (Rogers et al., 1983). The fertilization index (FI) was expressed as total number of sperm that penetrated the oocytes divided by total number of penetrated oocytes (Fig. 1).

#### (4) **Plasma membrane integrity (MI) and acrosome integrity (AI)**

Plasma membrane integrity (MI) was determined by hypo-osmotic swelling (HOS) test similar to that described by Jeyendran et al. (1984) for human sperm and later modified by Correa and Zavos (1994). Sodium citrate (0.73 g) and fructose (1.35 g) were dissolved in 100 ml distilled water (osmotic pressure ~150 mOsmol/kg) which constituted the HOS solution. To assess the sperm tail plasma membrane integrity, 50  $\mu$ l of the specimen containing spermatozoa was mixed with 500  $\mu$ l of pre-warmed hypo-osmotic solution (37 °C) for 30–45 min. A total of 200 spermatozoa were observed and assessed under a phase-contrast microscope at 400 $\times$  magnification. Swelling characterized by coiled tail indicated functional integrity of the plasma membrane. Determination of the percentage of live spermatozoa with intact acrosome (AI) followed the procedure described by Kovács and Foote (1992).

### 2.8. Seminal plasma protein comparison

Twenty samples from each method were used for the experiments, conducted in three replicates. The samples were thawed and pooled across bulls to avoid bull effect.

Seminal plasma protein profile comparison was performed by 2D-SDS-PAGE. All the chemicals used for 2D-SDS-PAGE were purchased from GE Healthcare (UK Ltd. Chalfont Buckinghamshire, UK).

#### 1) **Frozen-thawed seminal plasma extraction**

Frozen seminal plasma samples were thawed, re-centrifuged at 10,000  $\times$  g for 60 min at 4 °C. Protein was extracted in rehydration lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT]. Then the seminal plasma proteins were precipitated for 2 h at –20 °C with four volumes of ice-cold acetone and centrifuged for 15 min at 8000  $\times$  g, washed thrice with cold acetone followed by re-centrifugation to recover the pellet. The air-dried seminal plasma protein pellet was re-suspended in lysis buffer and re-centrifuged to remove any non-dissolved sample. The aliquots were stored at –80 °C until use. Seminal plasma samples were thawed and assayed for total protein concentrations using the modified Bradford assay with bovine serum albumin (1 mg/ml) as standards (Bradford, 1976).

#### 2) **Two-dimensional electrophoresis**

Twenty frozen seminal plasma samples (10 samples from each of the collection methods) were thawed at room temperature. Ten seminal plasma samples obtained either by RM or EE were pooled to give two main samples. Six 2-D electrophoresis gels (three replicates from each of the two main samples) were run. For analytical gels, each of the seminal plasma samples was adjusted to 200  $\mu$ g of total protein in 100  $\mu$ l of rehydration buffer solution [7 M urea, 2 M thiourea, 20 mM DTT, 0.5% (v/v) IPG buffer at pH 3–10]. Next, the mixture was cup-loaded onto their respective rehydrated 13 cm IPG strips with pH 3–10 linear (GE Healthcare; UK). Isoelectric focusing (IEF; first dimension) followed by second dimension SDS-PAGE in 15% polyacrylamide 13 cm-wide slab gels using a Ruby™ SE 600 system connected to a cooling bath system (Amersham Biosciences), were carried out at

20 °C. After the second dimension electrophoresis, proteins were visualized by staining with Blue–Silver Coomassie (Candiano et al., 2004). The gels were scanned with GS-800™ calibrated densitometer (Image scanner, Bio-Rad Laboratories Inc. USA) and quantitatively analysed using Image Master 2D Platinum 7.0 software (GE Healthcare; Amersham Bio Sciences, Upsala, Sweden).

### 3) Protein identification by MALDI-TOF-MS/MS

Some spots of interest were carefully excised and placed into micro tubes. Then, the spots were submitted to a service provider (Proteomics International Pty Ltd; Australia) to perform the trypsin digestion. The peptide mixture resulting from the protein digestion was added to the matrix used in the MALDI-TOF-MS/MS. The mass spectrum of the peptide mixture provided was matched with the Swiss-Prot and Ludwig-NR database to identify known proteins.

## 2.9. Statistical analysis

Comparison between the two semen collection methods (EE and RM) on normal morphology, plasma membrane and acrosome integrity, sperm capacitation, acrosome reaction and sperm motility parameters, sperm penetration assay and total protein content were done using Student's *t*-test of SPSS version 20. Seminal plasma proteins obtained by both collection methods were separated electrophoretically and analysed. To evaluate the protein composition of seminal plasma, 2D-SDS-PAGE was performed and relative protein intensity values were analysed using Student's *t*-test.

## 3. Results

Table 1 shows the effect of different semen collection methods on spermatozoa functionality after treatment with heparin and calcium ionophore A-23187 and the total protein content in frozen-thawed Bali bull seminal plasma. The AI, MI, AR, FP and FI were significantly higher ( $p < 0.05$ ) in samples collected via RM than EE. However, viability and normal morphology were unaffected by the method of collection. There was a significant increment observed in total protein of semen samples collected by EE compared with that collected by RM.

The effect of semen collection methods on motility parameters calculated by CASA system for frozen-thawed Bali bull semen after processing are shown in Table 2. A significant increment ( $p < 0.05$ ) was observed in general motility, progressive motility, VAP, VSL and VCL, collected by RM compared with those collected by EE.

Different protein profiles were observed between the two methods of semen collection (Fig. 2). Significant quantitative differences ( $p < 0.05$ ) were observed between collection methods (indicated by boxes labelled as 'a', 'b', 'c', 'd' and 'e'). Several spots (box c, with MW: 25–30; box d, with MW: 20–22 and box e, with MW: 12–16) showed higher ( $p < 0.05$ ) intensity in semen collected by EE (Figs. 2 and 3). Two protein spots in boxes labelled as 'a' and 'b' (a; MW: 37–45 kDa; and b; MW: 50–55 kDa) were only found in seminal plasma of semen collected via RM and these proteins were later identified by MALDI-TOF-MS/MS as phosphoglycerate kinase

**Table 1**

Effect of semen collection methods on total protein and spermatozoa functionality for frozen-thawed Bali bull semen treated with heparin and calcium ionophore A-23187.

Parameters <sup>a</sup>	Semen collection method	
	EE	RM
Total protein (g/100 ml)	3.68 ± 0.25 <sup>a</sup>	3.34 ± 0.19 <sup>b</sup>
Viability (%)	77.86 ± 2.69 <sup>a</sup>	78.64 ± 1.17 <sup>a</sup>
Motility (%)	71.44 ± 2.58 <sup>b</sup>	74.03 ± 2.25 <sup>a</sup>
NM (%)	68.24 ± 1.04 <sup>a</sup>	68.88 ± 1.15 <sup>a</sup>
AI (%)	62.86 ± 2.09 <sup>b</sup>	65.80 ± 1.88 <sup>a</sup>
MI (%)	55.85 ± 1.98 <sup>b</sup>	59.15 ± 1.62 <sup>a</sup>
AR (%)	60.23 ± 1.25 <sup>b</sup>	63.93 ± 1.08 <sup>a</sup>
FP (%)	73.64 ± 1.23 <sup>b</sup>	75.38 ± 1.79 <sup>a</sup>
FI	3.73 ± 0.40 <sup>b</sup>	4.31 ± 0.62 <sup>a</sup>

Data are means ± SEM. For each factor, different superscripts within rows differ significantly ( $p < 0.05$ ).

<sup>a</sup> Normal morphology (NM), acrosome integrity (AI), membrane integrity (MI), acrosome reaction (AR), fertilization percentage (FP), fertilization index (FI).

**Table 2**

Effect of semen collection methods on motility parameters calculated by CASA system for frozen-thawed Bali bull semen treated with heparin and calcium ionophore A-23187.

Parameters	Post-thawed evaluation after processing	
	EE	RM
General motility (%)	71.44 ± 2.58 <sup>b</sup>	74.03 ± 2.25 <sup>a</sup>
Progressive (%)	80.13 ± 2.31 <sup>b</sup>	83.66 ± 1.41 <sup>a</sup>
VAP (μm/s)	96.15 ± 2.62 <sup>b</sup>	99.82 ± 2.32 <sup>a</sup>
VSL (μm/s)	76.72 ± 0.99 <sup>b</sup>	78.03 ± 2.25 <sup>a</sup>
VCL (μm/s)	151.22 ± 3.74 <sup>b</sup>	156.12 ± 4.85 <sup>a</sup>
ALH (μm)	7.45 ± 0.22	7.94 ± 0.22
BCF (HZ)	28.42 ± 1.04	29.47 ± 0.98
STR (%)	79.80 ± 2.18	78.17 ± 2.47
LIN	50.73 ± 1.78	49.12 ± 1.16

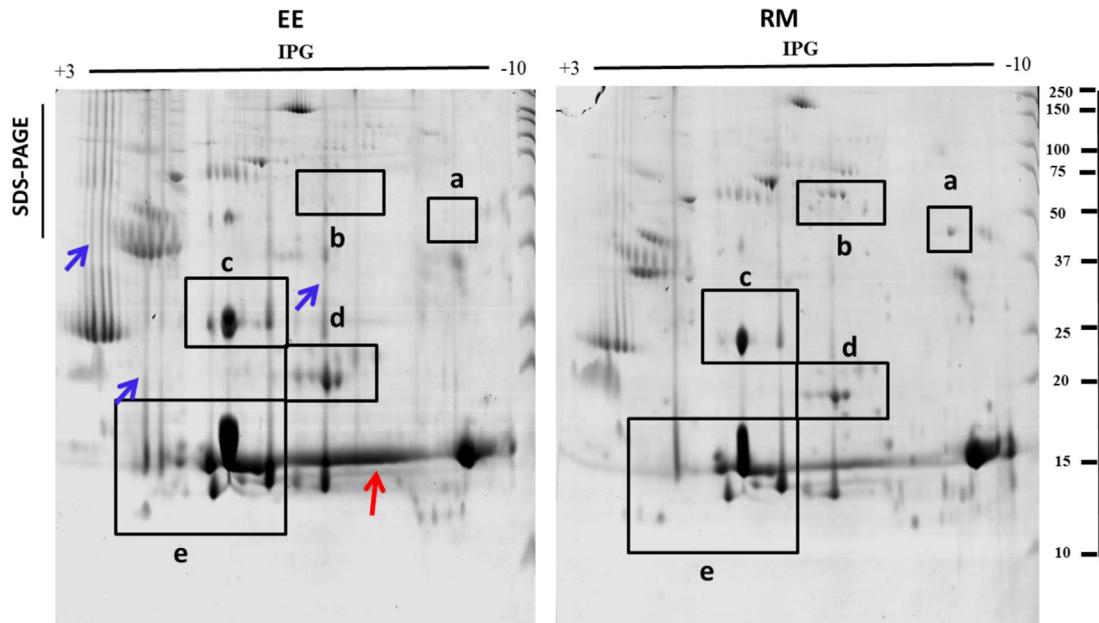
Data are means ± SEM. For each factor, different superscripts within rows differ significantly ( $p < 0.05$ ).

RM, transrectal massage; EE, electro-ejaculation; VAP, velocity average path; VSL, velocity according to the straight path; LIN, linearity index; ALH, amplitude of lateral head displacement; BCF, beat cross frequency; STR, straightness; LIN, linearity and AI, percentage of spermatozoa with intact acrosome; CASA, computer assisted semen analysis.

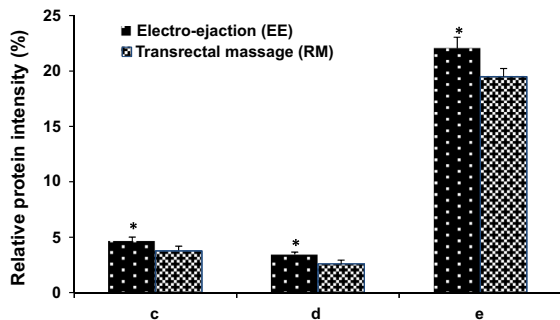
(PGK) and phospholipase A<sub>2</sub> (PLA<sub>2</sub>), respectively. About 52% of the total relative seminal plasma proteins (relative protein intensity %) were represented by four major protein fractions: 15.66% (MW: 37–45 kDa), 12.46% (MW: 25–30 kDa), 11.73% (MW: 14–16 kDa) 11.52% (MW: 12–15 kDa). The remaining 48% were represented by several minor proteins of low intensity. Table 3 shows ten proteins that were identified by two-dimensional electrophoresis and mass spectrometry MALDI-TOF-MS/MS. The predominant spots were < 30 kDa.

## 4. Discussion

Although results from the present study showed that frozen-thawed semen obtained by RM method produced significantly higher value for sperm quality parameters



**Fig. 2.** Representative two-dimensional polyacrylamide electrophoretic gel (2D-SDS PAGE) of Bali bull seminal plasma proteins from ejaculates obtained via electro-ejaculation (EE) and transrectal massage (RM). The line on the top shows the direction of the pH gradient from the basic end (+) to the acid end (-) in the first dimension from 3 to 10. Molecular weight markers and the corresponding weights are to the left (range from 250 to 10 kDa). Red and blue arrows are the horizontal and vertical lines respectively indicated high salt component that was found more in EE method. The box are as labelled as 'a', 'b', 'c', 'd' and 'e' showed proteins that had significant differences between the two collection methods. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 3.** Relative intensity protein content (mean  $\pm$  S.E.M of three replicates samples/method) in the three boxes (c, d and e), \* showed statistical difference ( $p < 0.05$ ).

(motility, AI, MI, AR, sperm parameters assessed individually by CASA and sperm penetration assay) compared to those collected by EE, the differences were small for many parameters. Bulls on RM method responded positively compared with those on EE. These findings supported earlier reports in rams (Quinn et al., 1968; Jiménez-Rabadán et al., 2012) and in dogs (Christensen et al., 2011), where spermatozoa collected by AV were more resistant to cold shock than those obtained by EE. However, other studies provided evidence that sperm quality after freezing was better for those sperm samples obtained by EE in rams (Ledesma et al., 2014; Marco-Jiménez et al., 2005) and elephant (O'Brien et al., 2013). These authors recorded higher number of stable and functional frozen-thawed spermatozoa (assessed for motility parameters, intact apical ridge, acrosomal status, plasma membrane

and capacitation status) from semen collected by EE. To our knowledge, there are no published reports comparing frozen-thawed semen parameters (AI, MI and AR), sperm penetration assay and sperm parameters assessed individually by CASA on ejaculates obtained through RM and EE.

The seminal fluid of mammals consists of various components of lipids, carbohydrates, and proteins. These are produced from various structures including the seminal vesicles, vas deferens, periurethral glands, epididymis, and prostate gland (Chandonnet et al., 1990; Okada et al., 2001). Several studies provide direct evidence that some proteins of the seminal plasma are adsorbed on to the surface of ejaculated sperm (Brewis and Gadella, 2010; Desnoyers and Manjunath, 1992; Manjunath and Thérien, 2002; Mentz et al., 1990). Earlier studies also showed that the seminal plasma proteins of mammals play a significant role in several essential steps such as sperm motility (Henricks et al., 1998; Kawano et al., 2004), viability by preserving the integrity of plasma membrane (Maxwell et al., 2007), sperm functionality such as regulating capacitation and gamete interaction and fusion (Killian et al., 1993; Bellin et al., 1998; Brandon et al., 1999; Villemure et al., 2003; Töpfer-Petersen et al., 2005). Individual proteins from the same family carry out their functions in a species-specific manner (Rodríguez-Martínez et al., 2011). Differences in their structures, relative abundance and patterns of expression appear to determine species-specific effects of homologous proteins (Calvete and Sanz, 2007). Seminal plasma proteins differ slightly in functionality related to their source, more clearly understood when fractionated ejaculates are examined (Rodríguez-Martínez et al., 2011). Therefore, a different proportion of seminal plasma could

**Table 3**

Proteins of the seminal plasma of Bali bull identified by two-dimensional electrophoresis and mass spectrometry MALDI-TOF-MS/MS.

Match ID (box)	GI accession number	Protein identification <sup>1</sup>	Peptide matches	Theoretical MW; pI <sup>2</sup>	Sequence coverage (%) <sup>3</sup>	Mascot MS/MS Score <sup>4</sup>	Experimental MW kDa; pI <sup>5</sup>	Collection method (EE or RM)
a	Q32KN6	Phosphoglycerate kinase	K.ELDYFSK.A K.LGDVYVNDAFGTAHRA	44,729;8.51	7	79	37–40; 8–8.5	RM
b	Q1RML9	Phospholipase A2–VII	R.GEEEFPLR.N K.IIGYLFTLK.G R.FQYPSNIIR.M R.IPQPLFFINSER.F K.YPLIIFSHGLGAFR.T R.GEEEFPLRNEQLR.Q R.GSVHQNFVDFTFATSK.I R.TGEKYPLIIFSHGLGAFR.T	50,519;6.12	17	605	50–55; 5.2–6	RM
c	P02784	Seminal plasma protein PDC-109	R.LGEDNINVVEGNEQFISASK.S K. HFDCTVHGSLFPWCSLDADYVGR. W	1,378;7.79	13	270	25–30; 5.3–5.6	
d	NP_776897.2	Tissue inhibitor of metalloproteases-2	R.IQYEIKQJK.M K.EVDSGNDIYGNPIKR.I	24,355;7.44	10	104	20–22;7.1–7.5	EE & RM
d	NP_776897.2	Tissue inhibitor of metalloproteases-2	R.IQYEIKQJK.M K.EVDSGNDIYGNPIKR.I	24,355;7.44	10	203	20–22;6.9–7.4	EE & RM
e	P02784	Seminal plasma protein PDC-109	K.CVFPFIYGGK.K K. HFDCTVHGSLFPWCSLDADYVGR. W	15,926;4.91	24	141	15–16; 5.4–5.8	EE & RM
e	P29392	Spermadhesion-1	K.ICEGSLMDYR.S K.TNCVWTIQMPPEYHVR.V K.YIREPEHPASFYEVLYFQDPQA K.ESLEIIEGPPESNSR.K	15,255;5.07	35	189	14–15; 5.2–5.5	EE & RM
e	P82292	Spermadhesion-Z13	K.ESLEIIEGPPESNSR.K	13,375;5.8	13	87	13–14; 5.5–5.8	EE & RM
e	P82292	Spermadhesion Z13	R.DVHLNCKNE R.KPNHPAPDFLIFR.R K.ESLEIIEGPPESNSR.K K.ESLEIIEGPPESNSR.K K.ICDTSHA EYTSCTNTMTVK.Y R.KICDTSHA EYTSCTNTMTVK.Y R. DVHLNCKNESLEIIEGPPESNSR.K	13,660; 5.8	23	193	13–14; 5.5–5.8	EE & RM
e	P02784	Seminal plasma protein PDC-109	K.CVFPFIYGGK.K K.CVFPFIYGGK.Y K. HFDCTVHGSLFPWCSLDADYVGR. W	15,926;4.91	25	181	14–16; 5–5.5	EE & RM

Note: proteins identified within this table were matched to *Bos taurus* rather than to *Bos javanicus* due limited protein database for *Bos javanicus*.

<sup>1</sup> Spot or train of spots as shown in 2D maps of Fig. 1.

<sup>2</sup> Theoretical molecular weight and pI represent the values given to the protein identified at the National Centre for Biotechnology Information non redundancy (NCBI NR) database.

<sup>3</sup> Sequence coverage as is given by Mascot.

<sup>4</sup> Probability-based Mowse score, according to Mascot. An individual ion score of at least 46 indicates identity or extensive homology ( $p < 0.05$ ). The protein score represents the sum of the unique ion scores when more than 1 precursor ion was used for MS/MS analysis.

<sup>5</sup> Experimental molecular weight and pI represent the values given to the protein spot. Proteins were visualized by Blue–Silver Coomassie staining.

be the cause of differences on sperm functionality and quality parameters that were observed in the different sperm collection methods in our study.

In this present study, the ejaculates collected via EE have greater total protein in the seminal plasma, which is supported by previous reports (Marco-Jiménez et al., 2005; Ledesma et al., 2014). The reason for the higher total protein content in the present study may be due to the effect of electrical stimuli on the accessory sex glands (Dziuk et al., 1954b; Pineda et al., 1987; Quinn and White, 1966) which resulted in voluminous secretions of seminal fluid (Ledesma et al., 2014; Sarsaifi et al., 2013) especially from the bulbourethral gland (Marco-Jiménez et al., 2008) and

seminal vesicles (Manjunath et al., 1987). These two glands are major protein contributors to bovine seminal plasma. Conversely, the protein composition of seminal plasma obtained by EE differed from that obtained by RM, as supported by Ledesma et al. (2014). The seminal plasma collected by EE in the present study contained low molecular weight proteins ( $\leq 30$  kDa) compared with that of RM. The protein profile of seminal plasma using 2D-PAGE had earlier been used for characterization of several proteins from seminal plasma of bulls (Aida et al., 1997; Asadpour et al., 2007; Killian et al., 1993), boars (Jonáková et al., 2007), stallions (Amann et al., 1987; Brandon et al., 1999) and rams (Jobim et al., 2005). According to Manjunath et al. (1987) the

major proteins in bovine seminal plasma contained a group of proteins of low molecular weight (14–30 kDa).

In the present study, the quantitative analysis of the 2D-PAGE protein maps identified a remarkable difference in percentages of protein fractions in various protein spots from the seminal plasma of semen samples collected via EE and RM. The present study also showed that the higher intensity spots with MW of 14–16 kDa have been identified as belonging to the family of bovine seminal plasma proteins (BSP). This proves that the collection method used has a significant effect on the total protein content in the seminal plasma. In addition, a group of proteins with different low molecular weights (boxes c, d and e as shown in Fig. 2) were found in the seminal plasma obtained by both methods, but higher intensity protein was observed in the 2D-PAGE of semen samples that were collected via EE.

The horizontal and vertical lines in the 2D-PAGE profile of semen samples obtained via EE indicated higher stimulation of seminal vesicles resulting in increased volume of ejaculates most probably containing sodium, potassium as well as the BSP proteins. The profiles also were observed to have more intense horizontal and vertical lines in EE than in RM. This may indicate presence of salt in the seminal plasma collected via EE. The result of the present study is supported by the findings of Quinn and White (1966). The higher concentrations of sodium and potassium found in seminal plasma of ejaculates collected via EE was due to a large volume of seminal plasma secretions (Quinn and White, 1966; Sarsaifi et al., 2013). In addition, Marco-Jiménez et al. (2008) showed that seminal plasma from ram sperm samples obtained by EE has higher Na<sup>+</sup> concentration compared with samples collected by AV.

The major protein fractions of bovine seminal plasma were represented by three acidic proteins, designated as BSP-A1/-A2, BSP-A3 (14–16 kDa) and BSP-30 kDa (collectively known as BSP proteins, which made up 40–50% of the total proteins in domestic bulls). These proteins are secretory products of the seminal vesicles and ampullae, and their biochemical characteristics have been well-described (Chacur, 2012; Moura et al., 2006a, 2006b).

From the mass spectrometry analysis seven proteins have been identified in the seminal plasma from EE ejaculates (boxes c, d and e; Figs. 2 and 3). Two proteins in boxes a and b with MW from 37–45 and 50–55 respectively have been determined as phosphoglycerate kinase (PGK) and phospholipase A<sub>2</sub> (PLA<sub>2</sub>) in the seminal plasma of semen obtained via RM.

Box 'a', which is similar to PGK, is a 415-residue metabolic enzyme that produces ATP. Phosphoglycerate kinase is a major enzyme used in glycolysis, in the first ATP-generating step of the glycolytic pathway (Dhar et al., 2010; Watson et al., 1982). The mammalian sperm must be highly motile for a long period to enable it to fertilize an oocyte. It has been suggested that ATP is required for sperm flagellar movement which is dependent predominantly on mitochondrial respiration. There are two pathways for ATP production in mammalian sperm: glycolysis and mitochondrial respiration (Mukai and Okuno, 2004).

Several studies have documented the relationship between glycolysis and capacitation-dependent cell signalling (Urner and Sakkas, 2003). Although the importance of

glycolysis for protein phosphorylation and fertilization has received recognition in recent years, it has not been associated with energy production for flagellar movement (Mukai and Okuno, 2004). Box 'b' has been shown to be homologous with PLA<sub>2</sub> in Bali bull seminal plasma collected by RM method. PLA<sub>2</sub> are enzymes that release fatty acids from the second carbon group of glycerol (Nicolas et al., 1997) and has various functional features. PLA<sub>2</sub> and is known also to be involved in the acrosome reaction and sperm-oocyte interaction (Yuan et al., 2003). It has been reported that group X of PLA<sub>2</sub> is thought to be involved in sperm capacitation in mice (Escoffier et al., 2010). Also, in mice, group III PLA<sub>2</sub> is involved in sperm maturation (Escoffier et al., 2010). However, there is also evidence that PLA<sub>2</sub> stimulates immune cells (Granata et al., 2005) and has antimicrobial activity in the seminal plasma (Bourgeon et al., 2004; Moura et al., 2007). In the present study, the ejaculates obtained via RM contained PGK and PLA<sub>2</sub> which may have contributed to the better sperm quality.

## 5. Conclusion

In conclusion, the present results suggest that the ejaculates of Bali bulls obtained by RM showed better in vitro sperm quality after freezing and thawing. Semen quality was found to be better in RM than EE samples after treatment with heparin and calcium ionophore A-23187. In addition some low molecular weight proteins were up-regulated in the seminal plasma obtained from the EE method. Finally, the most significant finding is that PGK and PLA<sub>2</sub> proteins are only present in the seminal plasma of semen collected via RM.

## Conflict of interest

We declare that there are no conflicts of interests in this project.

## Acknowledgements

This work has been supported by the Agro Biotechnology Institute (ABI), Ministry of Science Technology and Innovation, Malaysia (Research Project Number: 08/05/ABI-ab032). Our special thanks to FELDA farms and MARDI station in Pahang Malaysia.

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