Small Ruminant Research 144 (2016) 90–99

Contents lists available at ScienceDirect

Small Ruminant Research

journal homepage: www.elsevier.com/locate/smallrumres

Relationship between total protein concentration of seminal plasma and sperm characteristics of highly fertile, fertile and subfertile Barki ram semen collected by electroejaculation

Essam A. Almadaly[a,*], Foad A. Farrag[b], Islam M. Saadeldin[c], Mohammed A. El-Magd[b], Ibrahim M. Abd El-Razek[d]

[a] Department of Theriogenology, Faculty of Veterinary Medicine, Kafrelsheikh University, El-Geish Street, 33516 Kafrelsheikh, Egypt
[b] Department of Anatomy, Faculty of Veterinary Medicine, Kafrelsheikh University, El-Geish Street, 33516 Kafrelsheikh, Egypt
[c] Department of Physiology, Faculty of Veterinary Medicine, Zagazig University, 44519 Zagazig, Egypt
[d] Department of Animal production, Faculty of Agriculture, Kafrelsheikh University, El-Geish Street, 33516 Kafrelsheikh, Egypt

ARTICLE INFO

Article history:
Received 23 April 2016
Received in revised form 29 July 2016
Accepted 30 July 2016
Available online 1 August 2016

Keywords:
Ram
Fertility
Subfertility
SDS-PAGE
Total protein concentration (TPC)

ABSTRACT

The aim of this study was to determine the relationship between total protein concentration (TPC) of seminal plasma (SP) and semen characteristics in rams. A total of 105 ejaculates from 21 Barki rams, of known fertility (9 highly fertile, 9 fertile and 3 subfertile) were collected by electroejaculation (EE) and used in the present study. An aliquot (200 μl) from each ejaculate was used for evaluation of semen characteristics (wave motion, motility, viability, sperm morphology, intact-acrosome, sperm concentration and total sperm count per ejaculate). The remaining volume was centrifuged to collect SP and TPC (g/dl) of SP was estimated with hand-held Refractometer; then SP proteins were separated on 15% (w/v) polyacrylamide gel. The results revealed that TPC of SP and almost all semen characteristics were significantly ($P < 0.05$) lower in subfertile (SF) group than both highly fertile (HF) and fertile (F) groups but the longest (85.0 ± 4.1 s) stimulation time (ST) was recorded in SF group. Moreover, TPC of SP in HF and F groups showed highly significant ($P < 0.001$) negative correlation with ST but showed highly significant positive correlation with all semen characteristics. However, in SF group neither ST nor semen characteristics showed any significant correlation with TPC of SP. Eosin-nigrosin (EN) stain, hypoosmotic swelling test (HOST) and glutaraldehyde fixation (GF) were capable to discriminate among HF, F and SF groups of rams. In total, fourteen protein bands with different molecular weights, ranging from 7 to 91 kDa, were detected on polyacrylamide gel. Protein bands of 11, 13 and 22.5 kDa were more prominent in HF group than F group but it was faint to absent in SF group. Moreover, bands of 91, 80 and 27 kDa were prominent in F and SF groups than HF group. In conclusion, TPC and protein profile of SP were closely correlated with semen characteristics and might be useful tools to determine ram fertility or semen quality. Protein bands of 11, 13 and 22.5 kDa were fertility-associated proteins in Barki rams SP. The effects of these fertility-associated proteins on sperm cell function required further investigation.

© 2016 Elsevier B.V. All rights reserved.

1. Introduction

Most progress in improving reproductive efficiency can be made by accurate estimation of the male fertility and their selective use (Foote, 2003; Utt, 2016). Thereof, prediction of male fertility is an often sought-after endeavor for many species of domestic animals. The structural soundness, quality of semen, level of libido and plan of nutrition affect the male reproductive performance (Lindsay, 1996; Wheaton et al., 1992). Male fertility depends on series of factors which range from animal behavior and physical conditions, to features that are linked directly to the semen such as sperm motility, levels of specific spermatic membrane proteins and biochemical constituents of the seminal plasma (SP; Assumpcao et al., 2005).

Standard semen analysis has involved a number of parameters such as sperm concentration, motility, morphology, and sperm membrane integrity for the assessment of male fertility. Thereby, sperm motility, viability and morphology, percentage of intact-acrosome and hypoosmotic swelling test (HOST) responsive sperms have been extensively evaluated as an indication of

* Corresponding author.
E-mail addresses: dr_essam_2005@yahoo.com, habibysofy@yahoo.com (E.A. Almadaly).

http://dx.doi.org/10.1016/j.smallrumres.2016.07.023
0921-4488/© 2016 Elsevier B.V. All rights reserved.

sperm's ability to fertilize an oocyte (Kirk et al., 2005). More attention has been given to evaluate sperm membrane integrity because its proper function is essential for sperm metabolism, capacitation, ova binding, and acrosome reaction. Hence, assessment of plasma membrane integrity is considered to be useful for predicting the fertilizing ability of a sperm (Brito et al., 2003) and semen fertility appears to be more closely related to membrane integrity than other sperm characteristics (Bacinoglu et al., 2008; Santolaria et al., 2015).

Evidence suggests that SP which is a complex mixture of secretions from testis, epididymis and accessory sex glands contains factors that modulate the fertilizing ability of sperm (Aslam et al., 2014; Henalut et al., 1995). Since spermatozoa are in contact with SP, for a relatively short period, it was initially thought to serve as a transport medium, but it is now recognized to have crucial effects on many aspects of mammalian sperm cell functions including sperm motility (Maxwell et al., 2007), regulation of capacitation (Leahy and Gadella, 2011), sperm storage in the female reproductive tract (Talevi and Gualtieri, 2010) and modulation of the female immune response to tolerate spermatozoa and the conceptus (Robertson, 2007). These effects are directed by the multifunctional action of numerous inorganic and organic components especially SP proteins.

Despite the physiological significance of SP, its molecular composition is complex and the various proteins present in it are poorly understood. In the last decade, several SP proteins have been identified, isolated and characterized. Evidence suggests that SP proteins are different among species and some SP proteins are associated with fertility in various species. Previous studies are generally related to the comparisons of SP composition between males of different fertility (Yue et al., 2009) or the isolation and characterization of specific SP protein that could influence sperm capacitation and fertilization (Talevi and Gualtieri, 2010). The biochemical analyses of SP are used for semen evaluation because semen characteristics alone are not completely satisfactory for semen appraisal in the current practice of commercial artificial insemination (AI). Although, Yue et al. (2009) have proved that ovine SP contains specific proteins that are associated with fertility and sperm characteristics of semen sample collected by the aid of artificial vagina (AV). The relationship between total protein concentration (TPC) of SP and semen characteristics in rams with different fertility has been little studied. Therefore, the present study was conducted to: (1) find out the correlation, if any, between TPC of SP and semen characteristics in Barki rams with different fertility; (2) investigate the possibility that either glutaraldehyde fixation (GF) or eosin-nigrosin (EN) stain or HOST could discriminate among Barki rams with different fertility, and (3) separate and identify the fertility-associated proteins in ovine SP.

2. Materials and methods

2.1. Media

Unless otherwise stated, all chemicals used were obtained from Sigma–Aldrich Chemical Company (St. Louis, MO, USA), and the solutions were prepared using Milli-Q water (Merck Millipore, Darmstadt, Germany). The vital stain EN was prepared by dissolving 5 g eosin and 30 g nigrosin in 300 ml sodium citrate 2.9% (w/v), followed by filtration and then was stored at 4 °C until use. Freshly prepared 1% (v/v) glutaraldehyde in 0.165 M sodium cacodylate buffer, adjusted to pH 7.3 at 25 °C was used for evaluation of % intact-acrosome and % abnormal morphology. Sperm counting medium [0.9% (w/v) NaCl, 0.1% (v/v) formaldehyde, 0.1% (w/v) polyethylene glycol and 10 mM ethylenediaminetetraacetic acid (EDTA) in H_2O] was used for counting of sperm concentration.

The hypoosmotic solution (150 mOsm/l), was prepared by dissolving 7.35 g sodium citrate and 13.5 g fructose in 1000 ml H_2O (Jeyendran et al., 1984) and stored at 4 °C until use. Electrophoresis sample buffer [4% (w/v) sodium dodecyl sulphate (SDS), 20% (v/v) glycerol and 10% (v/v) β-mercaptoethanol, pH 6.8 at 25 °C], was used in electrophoresis of ovine SP proteins. Acrylamide and methylene bisacrylamide was purchased from Bioshop Company (Canada). SDS and other electrophoresis chemicals were of analytical grade. Coomassie brilliant blue (CBB) stain containing 0.1% (w/v) Coomassie brilliant blue R-250, 30% (v/v) methanol and 10% (v/v) acetic acid was used for detection of protein bands following electrophoresis.

2.2. Experimental animals

A total of 21 sexually mature and healthy Barki rams (3–4 years-old) kept at Sakha Animal Production Farm, Agricultural Research Center, Egypt were used in this study. This farm located in Kafrelsheikh (Northern Egypt, Latitude 31°N). All rams were kept separately in individual pens and fed a standard diet with water *ad libitum*. According to the field fertility (determined from records maintained at the study station) of rams in the previous breeding season they were selected and classified into three groups as following: (1) highly fertile (HF) group of average fertility rate above 80%, (2) fertile (F) group of average fertility rate between 60% and 80%, and (3) subfertile (SF) group of average fertility rate below 50%. The number of rams was nine in HF and F groups and although, we are deeply concerned about the increase of rams in SF group but unfortunately, there were only three subfertile rams in our farm. Thus, the number of animals in SF group was three rams only. All rams were ejaculated twice before the study was initiated and during the study five ejaculates were obtained from each ram with a minimum of 48 h elapsed between consecutive collections from any one ram. The study was carried out under the Animal Welfare Act regulations of Egypt guidelines with approval granted by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Kafrelsheikh University, Kafrelsheikh, Egypt.

2.3. Testicular volume (TV)

Using graduated caliper, testicular dimensions (length, width and thickness) were measured and TV was calculated according to Bailey et al. (1998).

2.4. Semen collection

Ejaculates were collected from all rams by electroejaculation (EE, 08:00-09:00 am) as previously described by Garde et al. (2003). Rams were restrained in recumbent position on table, the rectum was cleaned of feaces and the preputial area was shaved and washed with normal saline 0.9%, then thoroughly dried to prevent the contamination of semen. An ElectrojacIV® stimulator (Electrojac® IV, Germany) was used, with a rectal probe 17 cm long and 2.5 cm in diameter with three electrodes. The rectal probe was lubricated, gently inserted into rectum and orientated so that the electrodes were positioned ventrally. The ElectrojacIV® was used at automatic setting where the EE regime consisted of consecutive series of 5 s pulses of similar voltage, each separated by 5 s break. Each series consisted of a total of five pulses. The initial voltage was 1 V which was increased in each series until a maximum of 10 V. Unhygienic and ejaculates contaminated with urine were rejected. The elapsed time between initiation of electrical stimulation and ejaculation was recorded and designated as "stimulation time (ST)".

2.5. Semen evaluation

Immediately after collection, the ejaculate was visually examined for color, consistency and hygienic quality. After visual examination the ejaculate was placed in a 37 °C water bath and the ejaculate volume was recorded. From each ejaculate, an aliquot (200 μl) of semen was used for evaluation of the following sperm characteristics:

2.5.1. Sperm motility

Wave motion and motility % were evaluated immediately after collection by Hulet and Ercanbrack (1962) method. Briefly, wave motion was assessed by preparing a wet mount (2 μl) of the spermatozoa and examined under a phase-contrast microscope (100×, Hund, Germany) fitted with stage warmer and scored subjectively from 0 (no motion) to 5 (numerous rapid waves) on a scale with steps equal to 1 according to the original method described by Evans and Maxwell (1987). The percentage of motile spermatozoa was evaluated subjectively in aliquots of semen diluted 1:100 in 2.9% (w/v) warm sodium citrate, covered with cover slip (18 mm × 18 mm), and examined under a phase-contrast microscope (400×) fitted with stage warmer of 38.5 °C. Approximately, five fields per sample were examined by the same technician and motility was estimated in increments of 5%.

2.5.2. Sperm viability

The percentages of viable (% intact-membrane) and abnormal (% abnormal morphology) spermatozoa were estimated by examining at least 200 spermatozoa stained with EN stain (Mortimer, 1994). Briefly, an aliquot (2 μl) of semen was stained with 20 μl of warm EN stain, smeared onto a slide, and dried on warming plate. Stained smears were evaluated for percentages of intact-membrane and abnormal morphology under 1000× magnification with bright field-microscopy. Upon examination, the unstained sperm indicate intact-membrane sperm and the stained one indicate damaged-membrane sperm. The proportion of unstained sperm cells was expressed as viability %. Finally, % abnormal morphology obtained with EN stain was compared with those obtained with GF. For each ejaculate, all analyses were done in duplicate.

2.5.3. % intact-acrosome and % abnormal morphology

An aliquot of semen (5 μl), was diluted with 50 μl of 0.16 M NaCl, and spermatozoa were fixed with 1% (v/v) glutaraldehyde by mixing the diluted semen with an equal volume of 2% (v/v) glutaraldehyde/0.165 M sodium cacodylate buffer (pH 7.3 at 25 °C) at room temperature for a minimum of 30 min. After incubation, sperm samples were examined using a phase-contrast microscopy (1000×) for sperm morphology (% abnormal morphology) and acrosome integrity (% intact-acrosome), where spermatozoa showing a dense, thick apical ridge on the head were considered intact-acrosome (Shams-Borhan and Harrison, 1981); in total 400 spermatozoa were examined. Fixation of sperm cells using 1% glutaraldehyde in 0.165 M sodium cacodylate buffer and evaluation of % intact-acrosome and % abnormal morphology was designated as "GF" throughout the manuscript according to Almadaly et al. (2012).

2.5.4. Hypoosmotic swelling test (HOST)

To assess the functional status of sperm plasma membrane, the spermatozoa was subjected to HOST (Jeyendran et al., 1984, 1992). Briefly, HOST was performed by incubating an aliquot (100 μl) of semen sample with 1 ml of 150 mOsm hypoosmotic solution at 37 °C for 1 h. After incubation, an aliquot (50 μl), of the spermatozoa subjected to HOST was mixed with an equal volume of 2% glutaraldehyde/0.165 M sodium cacodylate buffer (pH 7.3 at 25 °C) at room temperature for a minimum of 30 min. Just prior to analysis the tubes were gently mixed and two wet mounts 2 μl each of glutaraldehyde fixed sperm cells were cover slipped and observed under a phase-contrast microscope at 1000× magnification. At least 200 spermatozoa on each mount were counted and the proportion of spermatozoa exhibits tail swelling or having coiled, bent and looped tails were determined (Y). In total 400 spermatozoa were examined in different microscopic fields. Proportion of HOST-positive sperm cells (% intact-membrane) was determined from the following equation (HOST-positive $\% = Y - X/400 \times 100$), where X was spermatozoa having tail abnormalities such as those shown in Fig. 3, panels L–P according to Fukui et al. (2004). Finally, % intact-membrane obtained with HOST was also compared with those obtained by EN stain.

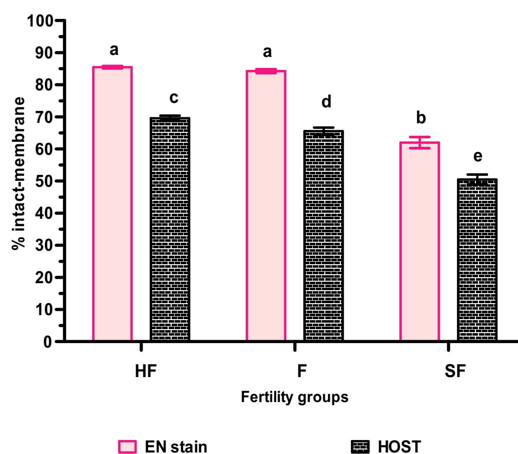

Fig. 1. Comparison of % intact-membrane between EN stain and HOST within the fertility groups.
All bars are mean ± SEM. Within the same fertility group and within the same evaluation method bars bearing different superscripts were significantly different at $P < 0.05$. HF = highly fertile; F = fertile; SF = subfertile; EN = eosin-nigrosin; HOST = hypoosmotic swelling test.

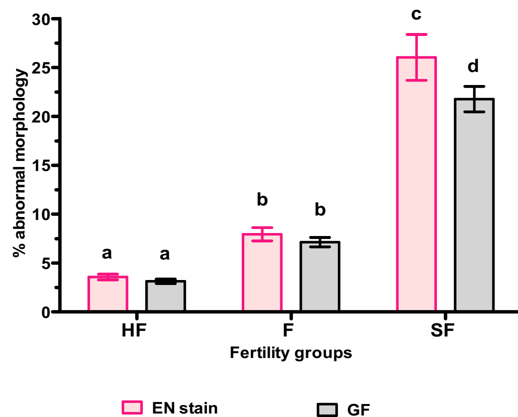

Fig. 2. Comparison of % abnormal morphology between EN stain and GF within the fertility groups.
All bars are mean ± SEM. Within the same fertility group and within the same evaluation method bars bearing different superscripts were significantly different at $P < 0.05$. HF = highly fertile; F = fertile; SF = subfertile; EN = eosin-nigrosin; GF = glutaraldehyde fixation.

2.5.5. Sperm concentration

Sperm concentration was calculated in duplicate using a Neubauer chamber (Marienfeld, Germany) according to the method of World Health Organization (1992). Briefly, semen was diluted at a ratio of 1:300 in sperm counting medium and an aliquot

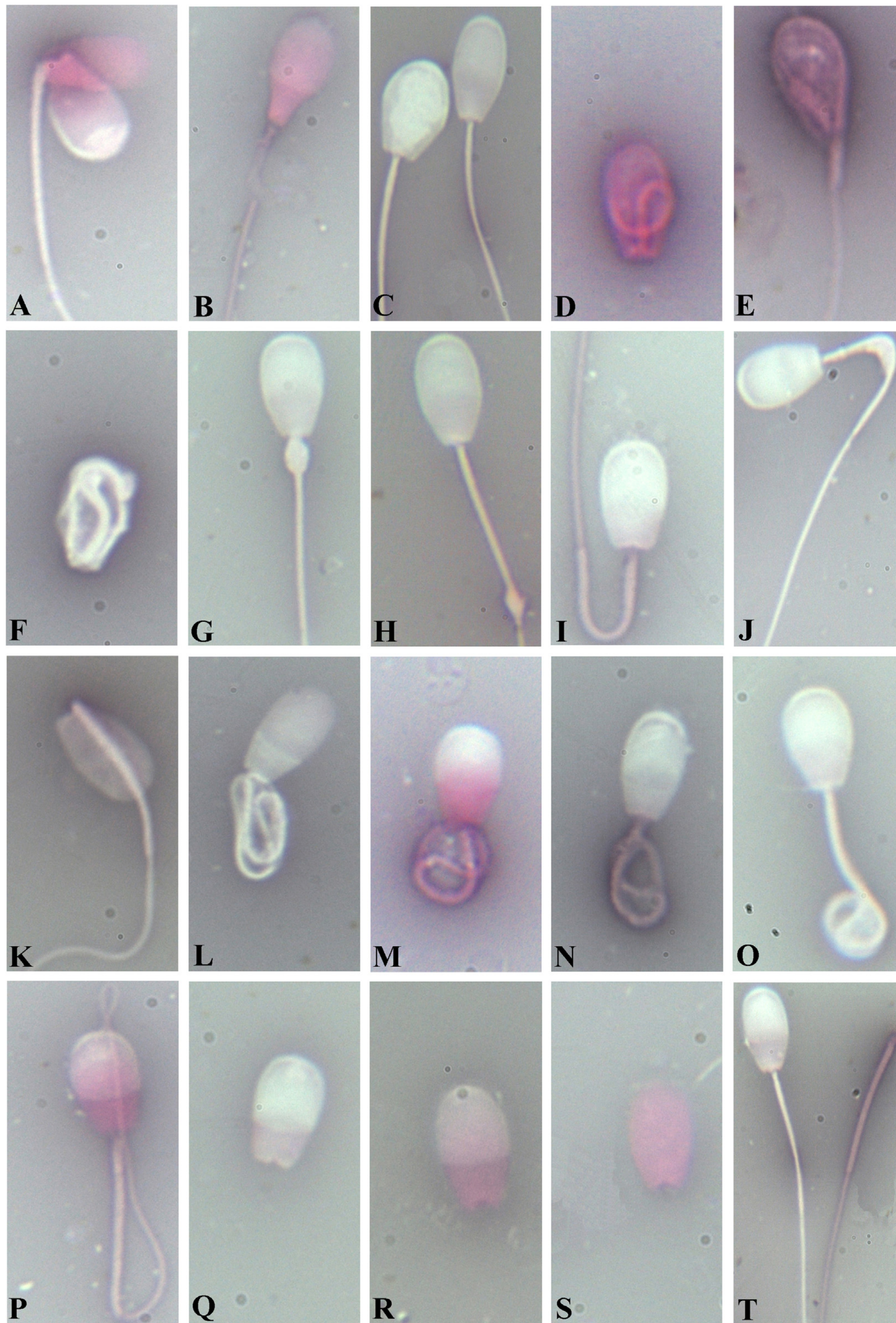

Fig. 3. The detected sperm abnormalities by EN stain and GF.
These abnormalities were visualized with a 1000 × phase-contrast objective. Double head (panel-A), pear shaped head (panel-B), macrocephalic sperm (panel-C), dag defect (panels-D–F), proximal protoplasmic droplet (panel-G), distal protoplasmic droplet (panel-H), bent middle-piece (panels-I,J), broken neck (panel-K), looped tail (panels-L–N), terminally coiled tail (panel-O), bent tail (panel-P), tailless head (panels-Q–S), detached tail (panel-T).

(12.5 μl) of mixture was placed on a haemocytometer chamber. The number of cells was counted under a phase-contrast microscope at 400× magnification. Sperm concentration was calculated and expressed as 10^9/ml. Finally, the total sperm count per ejaculate was calculated by multiplying ejaculate volume times the sperm concentration.

2.6. Recovery of SP and measuring its TPC

Immediately, after semen collection SP was obtained by centrifuging at 12000 × g for 30 min in a cooling centrifuge at 4 °C. The supernatants (SP) were transferred into 0.6 ml centrifuge tubes, and centrifuged again to eliminate the remaining cells and debris in order to obtain on clear SP. Finally, SP was stored at −80 °C until electrophoresis. The TPC (g/dl) of SP was determined using a handheld Refractometer (ATAGO, Brix 0–32%, Japan) according to Marsh and Fingerhut (1962).

2.7. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of ovine SP

Sodium dodecyl sulphate-PAGE was performed in five replicates using SP of seven (3 HF, 3 F and 1 SF) rams within the same gel to determine the molecular weight and the relative content of various SP proteins among the three fertility groups of rams. Frozen SP samples were thawed at room temperature and subjected to SDS-PAGE according to the method previously described by Laemmli, (1970). Briefly, SP samples were diluted 1:1 (v/v) with the sample buffer [4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol and of pH 6.8] and boiled for 5 min. After centrifugation at 10000 × *g* for 5 min at 4 °C, the supernatants containing the proteins were collected and stored at −80 °C until analysis. Extracted SP proteins were separated on 15% (w/v) polyacrylamide gel containing 0.1% (w/v) SDS at room temperature and 20 mA/gel using a mini protean III vertical slab gel electrophoresis apparatus (Bio-Rad Laboratories, USA). After electrophoresis, gels were immersed for few min in a freshly prepared pre-fixative solution of 20% (v/v) methanol, 7.5% (v/v) acetic acid in H_2O. Gels were stained with 0.1% (w/v) Coomassie brilliant blue R-250 at room temperature with gentle shaking for 1 h (Bürk et al., 1983); then destained in a freshly prepared solution of 30% (v/v) methanol, 10% (v/v) acetic acid in H_2O overnight with gentle shaking. Finally, the gels were scanned (Epson PM-A900, Japan) and the apparent molecular weight was estimated using broad-way dual prestained protein marker (ranging from 7 to 240 kDa, iNtRON Biotechnology, Korea).

2.8. Statistical analyses

The results were tabulated as mean ± standard error of mean (SEM). All analyses were carried out using a statistical software program (GraphPad Prism Version 5.0; GraphPad Software, San Diego, CA, USA). Analysis of variance (ANOVA) with Tukey's multiple comparison test was used subsequently for comparison of mean values at a significance level of P < 0.05. Pearson's correlation coefficient (Snedecor and Cochran, 1994) was applied to examine the relationship of TPC of SP with semen characteristics and the interrelationship among semen characteristics. Correlations with P < 0.05 were considered significant. The proportions of intact-membrane and abnormal morphology of the two evaluation methods were subjected to Student's paired *t*-test at a significance level of P < 0.05.

3. Results

3.1. Testicular volume, stimulation time and semen characteristics

The mean ± SEM of TV, ST and semen characteristics of the three fertility groups of rams have been summarized in Table 1. The obtained results revealed that TV was greater (P < 0.05) in HF group (430.4 ± 14.3 mm^3) than F (357.2 ± 24.6 mm^3) and SF (261.2 ± 8.4 mm^3) groups. All semen characteristics of SF group except ejaculate volume, sperm concentration and total sperm count per ejaculate were significantly (P < 0.05) lower than those of F and HF groups. Notably, all semen characteristics except ejaculate volume, sperm concentration and HOST-positive sperm cells were similar (P ≥ 0.05) between HF group and F group (Table 1). Furthermore, HF group ejaculated after short ST (38.4 ± 2.3 s) similar to those of F group (42.2 ± 1.6 s) whereas, SF group ejaculated after a prolonged (85.0 ± 4.1 s) ST. Also, SF group yielded lower (0.55 ± 0.02 ml) ejaculate volume compared with HF (1.27 ± 0.07 ml) group. Moreover, HF group had the highest proportions of motility (85.3 ± 0.5), viability (85.4 ± 0.3) and intact-acrosome (99.8 ± 0.1) without significant difference from F group but with highly significant (P < 0.001) difference than SF group. Interestingly, only the proportions of HOST-positive sperm cells showed highly significant (P < 0.001) difference among the three fertility groups. Also, F group yielded the highest sperm concentration (3.69 ± 0.23 × 10^9/ml) with significant difference from both HF (2.79 ± 0.25 × 10^9/ml) and SF (2.35 ± 0.29 × 10^9/ml) groups. Total sperm count per ejaculate of HF (4.03 ± 0.46) group was greater (P < 0.001) than that of SF (1.33 ± 0.19) group as shown in Table 1.

3.2. Correlation of the TPC of SP with ST and semen characteristics

Our data revealed that TPC (g/dl) of SP was similar in HF (5.9 ± 0.2) and F (5.8 ± 0.1) groups and both were greater (P < 0.001) than that of SF (3.8 ± 0.1) group. Regarding the correlation of the TPC of SP in each fertility group with the corresponding ST and semen characteristics our results revealed that in HF and F groups TPC of SP showed highly significant (P < 0.001) negative correlation with ST and showed highly significant (P < 0.001) positive correlation with all semen characteristics (ejaculate volume, wave motion, motility, viability, intact-acrosome, sperm concentration, total sperm count per ejaculate and HOST-positive sperm) as shown in Table 2. On the other hand, the TPC of SP in SF group did not have significant correlations with ST and semen characteristics (Table 2).

In regards to the overall correlation in the three fertility groups of rams the obtained data revealed that TPC of SP has highly significant negative (r = −0.721, P < 0.001) correlation with ST whereas, has highly significant (P < 0.001) positive correlation with TV and all semen characteristics as shown in Table 3. Likewise, TV showed significant negative correlation (r = −0.646, P < 0.01) with ST but has significant (P < 0.05) positive correlation with all semen characteristics except sperm concentration. Moreover, ST has highly significant (P < 0.001) negative correlation with all semen characteristics, whilst ejaculate volume has significant (P < 0.01) positive correlation with the other semen characteristics (Table 3). Wave motion has highly significant (P < 0.001) positive correlation with the proportions of motility (r = 0.836), viability (r = 0.858), intact-acrosome (r = 0.767) and HOST-positive sperm (r = 0.687) and motility has highly significant (P < 0.001) positive correlation with all semen characteristics. Viability showed highly significant (P < 0.001) positive correlation with proportions of intact-acrosome (r = 0.861) and HOST-positive sperm (r = 0.781) and intact-acrosome showed highly significant (r = 0.683, P < 0.001) positive correlation with HOST-positive sperm. Furthermore, sperm concentration revealed highly significant (r = 0.768, P < 0.001) positive correlation with total sperm count per ejaculate and HOST-positive sperm proportion was significantly (r = 0.515, P < 0.001) correlated with total sperm count per ejaculate (Table 3).

3.3. % intact-membrane by EN stain versus HOST

Fig. 1 shows that % intact-membrane of the three fertility groups of rams by EN stain were greater (P < 0.001) than those determined by HOST. Moreover, % intact-membrane of both HF and F groups was highly significant (P < 0.001) than that of SF group either by EN stain or HOST (Fig. 1). EN stain failed to detect a signifi-

Table 1
Mean ± SEM of TV, ST and semen characteristics in fresh ejaculates of Barki rams with different fertility.*

Fertility groups	No. of replicates (n)	TV (mm^3)	ST (second)	Ejaculate volume (ml)	Wave motion (0–5)	Motility (%)	Viability (%)	% intact-acrosome	Sperm concentration ($x10^9$/ml)	Total sperm count ($x10^9$)	HOST-positive (%)
HF group	45	430.4 ± 14.3^{a}	38.4 ± 2.3^{a}	1.27 ± 0.07^{a}	4.4 ± 0.1^{a}	85.3 ± 0.5^{a}	85.4 ± 0.3^{a}	99.8 ± 0.1^{a}	2.79 ± 0.25^{b}	4.03 ± 0.46^{a}	69.5 ± 0.7^{a}
F group	45	357.2 ± 24.6^{b}	42.2 ± 1.6^{a}	0.73 ± 0.03^{b}	4.7 ± 0.1^{a}	83.3 ± 0.7^{a}	84.2 ± 0.5^{a}	99.6 ± 0.1^{a}	3.69 ± 0.23^{a}	2.93 ± 0.27^{ab}	65.5 ± 1.1^{b}
SF group	15	261.2 ± 8.4^{b}	85.0 ± 4.1^{b}	0.55 ± 0.02^{b}	2.6 ± 0.1^{b}	57.6 ± 1.7^{b}	61.9 ± 1.7^{b}	92.8 ± 0.6^{b}	2.35 ± 0.29^{b}	1.33 ± 0.19^{b}	50.4 ± 1.5^{c}

n = 9 rams in HF and F groups but it was 3 rams in SF group with 5 ejaculates for each ram.
HF = highly fertile; F = fertile; SF = subfertile; TV = testicular volume; ST = stimulation time; HOST = hypoosmotic swelling test.
* Means bearing at least one common superscript in a column did not differ statistically ($P \geq 0.05$), otherwise significant at $P < 0.05$.

Table 2
Correlation of TPC (g/dl) of SP with ST and semen characteristics of Barki rams with different fertility.

Fertility groups	No. of replicates (n)	TPC of SP (g/dl)	ST	Ejaculate volume	Wave motion	Motility	Viability	Intact-acrosome	Sperm concentration	Total sperm count	HOST-positive
HF group	45	5.9 ± 0.2^{a}	-0.510^{***}	0.487^{***}	0.853^{***}	0.895^{***}	0.823^{***}	0.554^{***}	0.573^{***}	0.597^{***}	0.587^{***}
F group	45	5.8 ± 0.1^{a}	-0.733^{***}	0.586^{***}	0.707^{***}	0.616^{***}	0.592^{***}	0.556^{***}	0.881^{***}	0.762^{***}	0.537^{***}
SF group	15	3.8 ± 0.1^{b}	−0.115	0.457	0.078	0.218	0.158	0.129	0.385	0.463	0.504

n = 9 rams in HF and F groups but it was 3 rams in SF group with 5 ejaculates for each ram.
Upper values indicated Pearson's correlation (r) coefficients.
TPC = total protein concentration; HF = highly fertile; F = fertile; SF = subfertile; ST = stimulation time; HOST = hypoosmotic swelling test.
*** $P < 0.001$.

Table 3
Correlation matrix among TPC of SP, TV, and ST as well as semen characteristics of Barki rams with different fertility.

Parameter	TPC	TV	ST	Ejaculate volume	Wave motion	Motility	Viability	Intact-acrosome	Sperm concentration	Total sperm count	HOST-positive
TPC	1.000										
TV	0.862^{***}	1.000									
ST	-0.721^{***}	-0.646^{**}	1.000								
Ejaculate volume	0.530^{***}	0.712^{***}	-0.387^{***}	1.000							
Wave motion	0.795^{***}	0.536^{*}	-0.722^{***}	0.391^{***}	1.000						
Motility	0.751^{***}	0.644^{**}	-0.834^{***}	0.496^{***}	0.836^{***}	1.000					
Viability	0.707^{***}	0.629^{**}	-0.780^{***}	0.465^{***}	0.858^{***}	0.952^{***}	1.000				
Intact-acrosome	0.611^{***}	0.591^{**}	-0.763^{***}	0.375^{***}	0.767^{***}	0.843^{***}	0.861^{***}	1.000			
Sperm concentration	0.628^{***}	0.198	-0.491^{***}	0.269^{**}	0.470^{***}	0.418^{***}	0.340^{***}	0.253^{**}	1.000		
Total Sperm count	0.667^{***}	0.701^{***}	-0.512^{***}	0.786^{***}	0.472^{***}	0.521^{***}	0.453^{***}	0.355^{***}	0.768^{***}	1.000	
HOST- positive	0.702^{***}	0.662^{**}	-0.744^{***}	0.521^{***}	0.687^{***}	0.745^{***}	0.781^{***}	0.683^{***}	0.362^{***}	0.515^{***}	1.000

TPC = total protein concentration; TV = testicular volume; ST = stimulation time; HOST = hypoosmotic swelling test.
Upper values indicated Pearson's correlation (r) coefficients.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

cant difference in % intact-membrane between HF and F groups but HOST succeeded to detect a significant ($P < 0.05$) difference in % intact-membrane between HF and F groups as depicted in Fig. 1.

3.4. % abnormal morphology by EN stain versus GF

Fig. 2 shows that both EN stain and GF detect a highly significant ($P < 0.001$) difference in % abnormal morphology among HF, F and SF groups. Notably, HF group has the lowest proportion of abnormal spermatozoa either by EN stain (3.5 ± 0.3) or GF (3.1 ± 0.2). Also, % abnormal morphology of F group was 7.9 ± 0.7 and 7.1 ± 0.5 by EN stain and GF respectively, whereas, the highest proportion of abnormal spermatozoa was recorded for SF group either by EN stain (26.0 ± 2.3) or GF (21.7 ± 1.2). Within HF and F groups, % abnormal morphology was not significantly different between EN stain and GF but within SF group, % abnormal morphology by EN (26.0 ± 2.3) stain was greater ($P < 0.05$) than that determined with GF (21.7 ± 1.2). The detected forms of sperm abnormalities were summarized in Fig. 3.

3.5. Protein profile of ovine SP with different fertility

A total of fourteen protein bands were detected and their molecular weights ranged from 7 to 91 kDa as shown in Fig. 4A–C. Interestingly, protein bands of 11, 13 and 22.5 kDa were more prominent in HF group compared with F group but it was faint to absent in SF group. In general, protein band of 7 kDa was prominent in HF and F rams but it was faint to absent in SF rams. Moreover, protein bands of 91, 80 and 27 kDa were prominent in F and SF groups than HF group. Surprisingly, the protein profile of SP in SF group revealed undetectable protein bands except three clear protein bands of 91, 80 and 27 kDa (Fig. 4A–C).

4. Discussion

The ejaculates collected by EE presented values of volume, motility, viability, sperm concentration (Yue et al., 2009) and wave motion (David et al., 2015; Sarlós et al., 2013) similar to that recorded in ram semen collected by AV, showing that our protocol of EE is efficient in collection of a semen sample with quality comparable to those obtained by AV. Possibly, the hygienic condition and

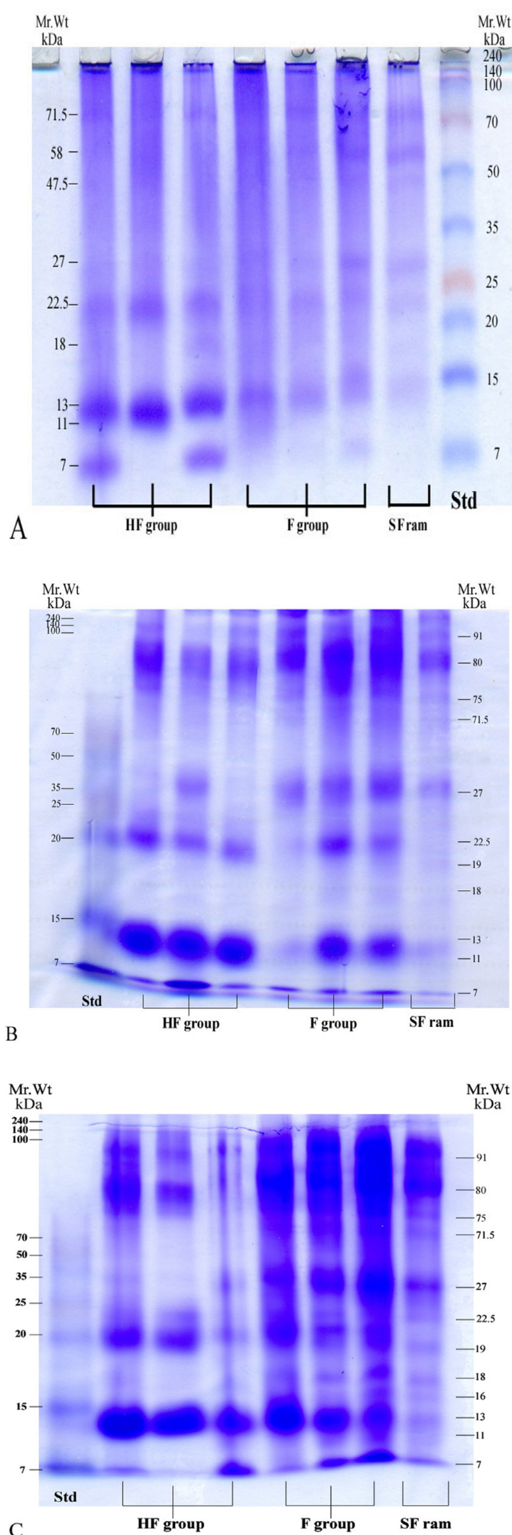

Fig. 4. SDS-PAGE of SP proteins in highly fertile, fertile and subfertile Barki rams. Coomassie brilliant blue-stained protein bands of ovine SP separated by SDS-PAGE; Std = molecular weight standards; HF = highly fertile; F = fertile; SF = subfertile; Mr. Wt = molecular weight; kDa = kilodalton. The three (A–C) polyacrylamide gels represent all experimental rams each gel represent protein patterns of 7 rams including 3 HF, 3 F and 1 SF.

minimal stress applied on rams during collection, have contributed to disappearance the differences in semen characteristics between AV and EE samples. Furthermore, a recent study (Jiménez-Rabadán et al., 2016) reported that the post-thaw sperm parameters of ram semen collected by EE were similar to those collected by AV.

Even though, several studies proved a correlation between SP proteins and male fertility in some species of domestic animals such as bull (Killian et al., 1993), ram (Jobim et al., 2005) and buck (Villemure et al., 2003), a little information is available regarding the relationship of TPC of SP with semen characteristics and fertility in rams. The current study was conducted to get some information in this field wherein, TPC values obtained herein were higher than the values given by Mahsud et al. (2013) in SP of Lohi rams semen collected by AV. This difference may be due to the different breed of ram (Barki versus Lohi), method of TPC estimation (Refractometer versus Biuret method) and method of semen collection (EE versus AV). The TPC make up the amphoteric property of SP and low protein content in SP reduces its buffering capacity (Mahsud et al., 2013) and in turns semen quality which might be the reason for worse semen quality of SF group. It is also important to emphasize that TPC of SP showed a highly significant ($P < 0.001$) positive correlation with all semen characteristics of Barki rams in agreement with the findings of Gundogan (2006) and to some extent corroborated well with the findings of Mahsud et al. (2013). Hence, our findings suggest that poor semen quality is associated with low TPC in SP and undetectable protein bands on polyacrylamide gel in accordance with Barrios et al. (2000). Furthermore, this study supports our recent investigation (Almadaly et al., 2015) that TPC of SP measured by the hand-held Refractometer are significantly correlated with those by the Lowry method ($r = 0.745$; $P < 0.002$). Accordingly, it has been concluded that both Refractmeter and Lowry methods measure the same parameter in SP. As well, HF group has higher TPC and denser protein bands especially fertility-associated proteins of 11, 13 and 22.5 kDa than those of F and SF groups. Based on the current findings, it is obvious that measuring TPC of SP with hand-held Refractometer could be used as useful tool to differentiate at least between F group and SF group of Barki rams.

In addition, ST has highly significant ($P < 0.001$) negative correlation with all semen characteristics which means that semen sample collected after a prolonged ST has poor semen quality compared to that collected after a short ST. This finding seems beneficial during breeding soundness evaluation of rams before the breeding season to exclude those ejaculated after a prolonged time of electrical stimulation due to their poor semen quality. In fact, the fertility of rams is basically characterized by the quality parameters of the ejaculate and the fertilization process consists of several events that all must occur efficiently to achieve successful conception. Hence, sperms which are defective, can affect any of these events leading to reduced fertility (Leahy and Gadella, 2011; Vicente-Fiel et al., 2014). These findings coincided with our results because all semen characteristics of SF group especially wave motion (2.6 ± 0.1), and proportions of motility (57.6 ± 1.7), viability (61.9 ± 1.7) intact-acrosome (92.8 ± 0.6) and HOST-positive (50.4 ± 1.5) were lower ($P < 0.001$) than those of F and HF groups. Furthermore, SF group has greater % abnormal morphology than F and HF groups wherein, the most common sperm abnormalities in SF group were primary sperm abnormalities such as pear shaped head (Fig. 3, panel B) and macrocephalic head (Fig. 3, panel C) and secondary sperm abnormalities such as dag defect (Fig. 3, panels D–F), proximal protoplasmic droplet (Fig. 3, panel G) and distal protoplasmic droplet (Fig. 3, panel H).

When evaluating semen, the ultimate goal is to accurately predict its fertilizing potential. The sperm membrane integrity is a critical event for sperm-oocyte interaction and fertilization (Brito et al., 2003). The present manuscript evaluates the plasma mem-

brane integrity of sperm cell by using vital stain (EN) and HOST to give an accurate diagnosis on the fertilizing potential of ram semen. There was clear difference between results of EN stain and HOST in the current study, because the proportions of intact-membrane sperm identified by HOST being consistently lower ($P < 0.001$) than those determined with EN stain within the same fertility group of rams. This result is consistent with the concept that vital stains are commonly used to evaluate the physical integrity of plasma membrane, while HOST evaluates the biochemical activity of plasma membrane; thereby the physical intactness of plasma membrane does not ensure that it is functional in agreement with similar studies done in stallions (Neild et al., 1999), boars (Zou and Yang, 2000) and bulls (Brito et al., 2003). This might be the reason for that HOST succeeded to discriminate among the three fertility groups of rams in terms of % intact-membrane but EN stain failed to discriminate between HF and F rams. In addition, the proportion of HOST-positive sperm was highly ($r = 0.781$, $P < 0.001$) correlated with the proportion of unstained sperm detected by EN stain.

Assays that determine if a sperm is alive, in particular viability and motility assays appear to most informative, as they consistently appear in multivariate correlations with male fertility. A recent study (David et al., 2015) reported that mass sperm motility is a convincing indicator of fertility in sheep. We found the values of wave motion and the proportions of motility, viability; intact-acrosome and HOST-positive sperm in HF and F groups have highly significant positive correlation with TPC of SP. In other words, all these semen characteristics should be positively correlated with ram fertility because all the above-mentioned semen characteristics were non-significantly correlated with TPC of SP in SF group. It has been proven that the proportion of HOST-positive sperm cells varies with animal; wave motion, progressive motility, sperm count, % intact-acrosome (Prasad et al., 1999) and individual fertility level (Jeyendran et al., 1984). Depending upon our obtained data which revealed the proportion of HOST-positive sperm cells has highly significant ($P < 0.001$) positive correlation with TPC of SP in F and HF groups. Consequently, it is obvious that the high TPC of SP is associated with good semen quality and fertility at least in F and HF Barki rams.

The breadth of *in vitro* laboratory evaluations of semen ranges from subjective microscope evaluation to proteomics. Microscopic evaluation of sperm motility and morphology have been, and will likely continue to be, two of the most important predictors of fertility in terms of utilization and the variation in fertility that these variables explain. The frequency of sperm abnormalities of various types have been correlated with fertility (Al-Makhzoomi et al., 2008; Gillan et al., 2008). Even though, within HF group and F group there was no significant difference in % abnormal morphology between EN stain and GF but within SF group the proportion of abnormal sperm cells was greater ($P < 0.05$) by EN stain than GF. Furthermore, the two methods could discriminate among the three fertility groups of rams depending upon the proportion of abnormal sperm cells. However, GF failed to detect any significant difference in % intact-acrosome between fertile and subfertile Japanese Black bull spermatozoa in our recent study (Almadaly et al., 2012). Fortunately, in the current study GF did not only succeed to detect a significant difference in % intact-acrosome between fertile and subfertile rams, but also it detects a significant difference in % abnormal morphology among highly fertile, fertile and subfertile rams. The reason for this discrepancy might be the type of spermatozoa (fresh or frozen-thawed), animal species (ovine or bovine) and method of semen collection (EE or AV); frozen-thawed bull semen collected by AV (Almadaly et al., 2012) versus fresh ram semen collected by EE in the current study.

According to Bergeron et al. (2005) most proteins in ovine SP are below 30 kDa, prevailing those with a molecular mass of 15–16 kDa and 22–24 kDa. These findings are consistent with our results because protein bands of 11, 13 and 22.5 kDa were predominant especially in F and HF groups and these protein bands represent the major proteins in ovine SP according to the size and the staining intensity of these bands. Our study might be unique in detection of a small molecular weight protein of 7 kDa which may be a new candidate of fertility-associated protein in ovine SP because it was barely detectable in SF rams. This low molecular weight protein (7 kDa) might be present only in SP collected by EE because recently Ledesma et al. (2014) found that EE increases low molecular weight proteins in ram SP. It is noteworthy that protein bands of 11, 13 and 22.5 kDa were predominant in HF group compared with F group but these protein bands were faint to absent in SF group. Accordingly, it was suggested that protein bands of 11, 13 and 22.5 kDa could be a kind of fertility-associated proteins in ovine SP where those of 11 and 13 kDa were similar to bovine SP proteins A1/A2 according to Jobim et al. (2005). Protein band of 13 kDa may correspond to spermadhesin of 15.5 kDa (≈45% of the total protein) and protein bands of 11, 18 and 22.5 kDa may correspond to proteins of 15, 22 and 24 kDa respectively in ram SP according to Bergeron et al. (2005).

Table 4
Protein bands detected in the present study with reference to known SP proteins.

Estimated molecular weight (kDa)	Candidate protein (molecular weight, kDa)
91	Prominin-2 (88)
80	Melanotransferrin (81)
75	Lactoferrin (77)
71.5	Serum albumin precursor (69)
58	Extracellular matrix protein 1 isoform 1 (60)
47.5	Phosphoglycerate kinase 2 (45)
27	14-3-3 protein zeta (26) or lipocalin-type PGDS (27)
22.5	Prostaglandin-H2-NULL-isomerase precursor (21)
19	Binder of sperm 5 (BSP5, 18)
18	Binder of sperm 5 (BSP5, 18)
16	Epididymal secretory protein E1 (16)
13	Spermadhesin Z13 (SPADH2, 15) or binder of sperm 1 (BSP1, 15)
11	Bodhesin-2 (12)
7	Caltrin-like protein (9)

PGDS = prostaglandin D_2 synthase; kDa = kilodalton.

A recent proteomic study (Soleilhavoup et al., 2014) using high resolution strategies has been identified more than 700 proteins in ram SP, including a high abundance of binder of sperm family proteins, the spermadhesin family, lactoferrin and newly identified proteins. It is of interest to note that, all the fourteen protein bands detected in our study were present within the major 40 proteins identified by Soleilhavoup et al. (2014). In the present study, the detected SP proteins of 91, 80, 75, 71.5, 58, 47.5, 18, 16 and 7 kDa (Fig. 4A–C) may correspond to prominin-2, melanotransferrin, lactoferrin, serum albumin precursor, extracellular matrix protein 1 isoform 1, phosphoglycerate kinase 2, binder of sperm 5, epididymal secretory protein E1 and caltrin-like protein, respectively, as summarized in Table 4 according to Soleilhavoup et al. (2014). Additionally, the predominant SP proteins of 27, 22.5, 13 and 11 kDa (Fig. 4A–C) may correspond to 14-3-3 protein zeta, prostaglandin-H2-NULL-isomerase precursor, spermadhesin Z13 or binder of sperm 1 and bodhesin-2, respectively (Soleilhavoup et al., 2014).

Prostaglandin D_2 synthase (PGDS) is a major epididymal secretory protein identified in several species (Fouchécourt et al., 1999, 2002; Gerena et al., 2000). This lipocalin-type PGDS identified in ram SP originates from epididymis not from prostate or vesicular glands (Fouchécourt et al., 1999) and showed a high capacity to bind to lipophilic molecules such as retinoic acid and testosterone (Fouchécourt et al., 1999, 2002). This high capacity of PGDS to bind and, potentially, transport steroid suggests that it could be involved

in the regulation and maintenance of functions of the epididymal epithelium. Thus, suggesting a possible role of PGDS in sperm maturation and/or storage. Taken together, these results suggest that protein band of 27 kDa detected in the present study may correspond to this 27 kDa lipocalin-type PGDS because the intensity of this band was different among the three fertility groups of rams. Moreover, in SF group this 27 kDa protein band was faint in comparison to HF and F groups of rams which might be responsible for the higher % abnormal morphology in SF group particularly secondary sperm abnormalities (dag defect, proximal and distal protoplasmic droplets). Although, the functions of several SP proteins are uncertain but in ram SP proteins have been proven to protect spermatozoa from cold shock prior to cryopreservation (de Graaf et al., 2008; Muino-Blanco et al., 2008). The other impacts of SP proteins on sperm cell function warranted further investigation to gain further insights into the potential roles of SP proteins in male reproduction.

5. Conclusions

Differences in SP protein composition of Barki rams with different fertility and fertility-associated proteins (11, 13 and 22.5 kDa) in ovine SP were detected. The TPC of SP was positively correlated with TV and all semen characteristics but negatively correlated with ST. Hence, TPC of SP might be an indicator of fertility in ram. Moreover, it has the advantage of being inexpensive, easy to conduct and rapid to perform and thus can be used routinely as an essential index to evaluate ram fertility and semen quality. All of GF, EN stain and HOST might be useful tools to differentiate among rams with different fertility. Further studies on several hundred ejaculates will aim to construct reliable and simple assay for the prediction of male fertility through SP protein analysis.

Conflict of interest statement

All authors declare that they have no relationship with people or organizations that could prejudice or bias the content of this paper.

Acknowledgments

The assistance of the staff members of Sakha Animal Production Farm is highly appreciated. This study was financially supported by Grant-in-Aid from the Egyptian government, Ministry of Higher Education, Egypt.

References

Almadaly, E., El-Kon, I., Heleil, B., Fattouh, E., Mukoujima, K., Ueda, T., Hoshino, Y., Takasu, M., Murase, T., 2012. Methodological factors affecting the results of staining frozen–thawed fertile and subfertile Japanese Black bull spermatozoa for acrosomal status. Anim. Reprod. Sci. 136, 23–32.

Almadaly, E., Hoshino, Y., Ueta, T., Mukoujima, K., Shukry, M., Farrag, F., El-Kon, I., Kita, K., Murase, T., 2015. Desalted and lyophilized bovine seminal plasma delays induction of the acrosome reaction in frozen-thawed bovine spermatozoa in response to calcium ionophore. Theriogenology 83, 175–185.

Al-Makhzoomi, A., Lundeheim, N., Haard, M., Rodriguez-Martinez, H., 2008. Sperm morphology and fertility of progeny-tested AI dairy bulls in Sweden. Theriogenology 70, 682–691.

Aslam, M.K., Kumaresan, A., Sharma, V.K., Tajmul, M., Chhillar, S., Chakravarty, A.K., Manimaran, A., Mohanty, T.K., Srinivasan, A., Yadav, S., 2014. Identification of putative fertility markers in seminal plasma of crossbred bulls through differential proteomics. Theriogenology 82 (9), 1254–1262, e1.

Assumpcao, T.I., Torres Jr., R.A.A., Sousa, M.V., Ricart, C.A.O., 2005. Correlation between fertility and levels of protein, sugar and free amino acids in seminal plasma of Nelore bulls. Arq. Bras. Med. Zootec. 57 (1), 123–129.

Bacinoglu, S., Tas, M., Cirit, U., Ozdas, O.B., Ak, K., 2008. The potential fertility estimation capacity of the hypoosmotic swelling test, the thermal stress test and a modified cervical mucus penetration test in the bovine. Anim. Reprod. Sci. 104, 38–46.

Bailey, T.L., Hudson, R.S., Powe, T.A., Riddell, M.G., Wolfe, D.F., Carson, R.L., 1998. Caliper and ultrasonographic measurements of bovine testicles and a mathematical formula for determining testicular volume and weight *in vivo*. Theriogenology 49, 581–594.

Barrios, B., Perez-Pe, R., Gallego, M., Tato, A., Osada, J., Muino-Blanco, T., Cebrian-Perez, J.A., 2000. Seminal plasma proteins revert the cold-shock damage on ram sperm membrane. Biol. Reprod. 63, 1531–1537.

Bergeron, A., Villemure, M., Lazure, C., Manjunath, P., 2005. Isolation and characterization of the major proteins of ram seminal plasma. Mol. Reprod. Dev. 71, 461–470.

Brito, F.C., Barth, A.D., Bilodeau-Goessels, S., Panich, P.L., Kastelic, P., 2003. Comparison of methods to evaluate the plasmalemma of bovine sperm and their relationship with *in vitro* fertilization rate. Theriogenology 60, 1539–1551.

Bürk, R.R., Eschenbruch, M., Leuthard, P., Steck, G., 1983. Sensitive detection of proteins and peptides in polyacrylamide gels after formaldehyde fixation. Meth. Enzymol. 91, 247–254.

David, I., Kohnke, P., Lagriffoul, G., Praud, O., Plouarboué, F., Degond, P., Druart, X., 2015. Mass sperm motility is associated with fertility in sheep. Anim. Reprod. Sci. 161, 75–81.

de Graaf, S.P., Leahy, T., Marti, J., Evans, G., Maxwell, W.M., 2008. Application of seminal plasma in sex-sorting and sperm cryopreservation. Theriogenology 70, 1360–1363.

Evans, G., Maxwell, W.M.C., 1987. Handling and examination semen. In: Salamon's Artificial Insemination of Sheep and Goats. Butterworths, Sydney, pp. 93–106.

Foote, R.H., 2003. Fertility estimation: a review of past experience and future prospects. Anim. Reprod. Sci. 75, 119–139.

Fouchécourt, S., Charpigny, G., Reinaud, P., Pascal Dumont, P., Dacheux, J.-L., 2002. Mammalian lipocalin-type prostaglandin D_2 synthase in the fluids of the male genital tract: putative biochemical and physiological functions. Biol. Reprod. 66, 458–467.

Fouchécourt, S., Dacheux, F., Dacheux, J.L., 1999. Glutathione-independent prostaglandin D2 synthase in ram and stallion epididymal fluids: origin and regulation. Biol. Reprod. 60, 558–566.

Fukui, Y., Togawa, M., Abe, N., Takano, Y., Asada, M., Okada, A., Iida, K., Ishikawa, H., Ohsumi, S., 2004. Validation of the sperm quality analyzer and the hypo-osmotic swelling test for frozen-thawed ram and minke whale (*Balanenoptera bonarensis*) spermatozoa. J. Reprod. Dev. 50, 147–154.

Garde, J.J., Soler, A.J., Cassinello, J., Crespo, C., Malo, A.F., Espeso, G., Gomendio, M., Roldan, E.M.S., 2003. Sperm cryopreservation in three species of endangered gazelles (*Gazella cuvieri*, *G. dama* mhorr, and *G. dorcas* neglecta). Biol. Reprod. 69, 602–611.

Gerena, R.L., Irikura, D., Eguchi, N., Urade, Y., Killian, G.J., 2000. Immunocytochemical localization of lipocalin-type prostaglandin D synthase in the bull testis and epididymis and on ejaculated sperm. Biol. Reprod. 62, 547–556.

Gillan, L., Kroetsch, T., Maxwell, W.M.C., Evans, G., 2008. Assessment of *in vitro* sperm characteristics in relation to fertility in dairy bulls. Anim. Reprod. Sci. 103, 201–214.

Gundogan, M., 2006. Some reproductive parameters and seminal plasma constituents in relation to season in Akkaraman and Awassi rams. Turk. J. Vet. Anim. Sci. 30, 95–100.

Henalut, M.A., Killian, G.J., Kavanaaugh, J.F., Gril, L.C., 1995. Effect of accessory sex gland fluid from bulls of different fertilities on the ability of cauda epididymal sperm to penetrate zona-free ovine oocytes. Biol. Reprod. 52, 390–397.

Hulet, C.V., Ercanbrack, S.K., 1962. A fertility index for ram. J. Anim. Sci. 21, 489–493.

Jeyendran, R.S., Van der Ven, H.H., Perez-Pelaez, M., Crabo, B.G., Zaneveld, L.J.D., 1984. Development of an assay to assess the functional integrity of the human sperm membrane and its relationship to other semen characteristics. J. Reprod. Fertil. 70, 219–228.

Jeyendran, R.S., Van der Ven, H.H., Zaneveld, L.J.D., 1992. The hypoosmotic swelling test: an update. Arch. Androl. 29, 105–116.

Jiménez-Rabadán, P., Soler, A.J., Ramón, M., García-Álvarez, O., Maroto-Morales, A., Iniesta-Cuerda, M., Fernández-Santos, M.R., Montoro, V., Pérez-Guzmán, M.D., Garde, J.J., 2016. Influence of semen collection method on sperm cryoresistance in small ruminants. Anim. Reprod. Sci. 167, 103–108.

Jobim, M.I.M., Oberst, E.R., Salbego, C.G., Wald, V.B., Horn, A.P., Mattos, R.C., 2005. BSP A1/A2 Like proteins in ram seminal plasma. Theriogenology 63, 2053–2062.

Killian, G.J., Chapman, D.A., Rogowski, L.A., 1993. Fertility-associated proteins in Holstein bull seminal plasma. Biol. Reprod. 49, 1202–1207.

Kirk, E.S., Squires, E.L., Graham, J.K., 2005. Comparison of *in vitro* laboratory analyses with the fertility of cryopreserved stallion spermatozoa. Theriogenology 64, 1422–1439.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leahy, T., Gadella, B.M., 2011. Sperm surface changes and physiological consequences induced by sperm handling and storage. Reproduction 142, 759–778.

Ledesma, A., Manes, J., Cesari, A., Alberio, R., Hozbor, F., 2014. Electroejaculation increases low molecular weight proteins in seminal plasma modifying sperm quality in Corriedale rams. Reprod. Domest. Anim. 49, 324–332.

Lindsay, D.R., 1996. Environment and reproductive behavior. Anim. Reprod. Sci. 42, 1–12.

Mahsud, T., Jamil, H., Qureshi, Z.I., Asi, M.N., Lodhi, L.A., Waqas, M.S., Ahmad, A., 2013. Semen quality parameters and selected bio-chemical constituents level in plasma of Lohi rams. Small Rumin. Res. 113, 175–178.

Marsh, W.H., Fingerhut, B., 1962. Serum protein determination by automatic recording refractometry. Clin. Chem. 8 (6), 640–646.
Maxwell, W.M.C., de Graaf, S.P., El-Hajj Ghaoui, R., Evans, G., 2007. Seminal plasma effects on sperm handling and female fertility. In: Juengle, J.L., Murray, F., Smith, F. (Eds.), Reproduction in Domestic Ruminants VI, 64. Nottingham University Press, Nottingham, UK, pp. 13–37.
Mortimer, D., 1994. Sperm recovery techniques to maximize fertilizing capacity. J. Reprod. Fertil. Dev. 6, 25–31.
Muino-Blanco, T., Perez-Pe, R., Cebrian-Perez, J.A., 2008. Seminal plasma proteins and sperm resistance to stress. Reprod. Domest. Anim. 43, 18–31.
Neild, D., Chaves, G., Flores, M., Mora, N., Beconi, M., Aguero, A., 1999. Hypoosmotic test in equine spermatozoa. Theriogenology 51, 721–727.
Prasad, J.K., Kumar, S., Mohan, G., Shanker, U., Agarwal, S.K., 1999. Hypo-osmotic swelling test (HOST) and its response in fresh and freeze-thawed semen. Indian J. Anim. Sci. 69, 766–769.
Robertson, S.A., 2007. Seminal fluid signaling in the female reproductive tract: lessons from rodents and pigs. J. Anim. Sci. 85, 36–44.
Santolaria, P., Vicente-Fiel, S., Palacín, I., Fantova, E., Blasco, M.E., Silvestre, M.A., Yániz, J.L., 2015. Predictive capacity of sperm quality parameters and sperm subpopulations on field fertility after artificial insemination in sheep. Anim. Reprod. Sci. 163, 82–88.
Sarlós, P., Egerszegi, I., Balogh, O., Molnár, A., Cseh, S., Rátky, J., 2013. Seasonal changes of scrotal circumference, blood plasma testosterone concentration and semen characteristics in Racka rams. Small Rumin. Res. 111, 90–95.
Shams-Borhan, G., Harrison, R.A.P., 1981. Production, characterization, and use of ionophore-induced: calcium- dependent acrosome reaction in ram spermatozoa. Gamete Res. 4, 407–432.
Snedecor, G.W., Cochran, W.G., 1994. Statistical Methods, 9th edition. Iowa State University Press Ames, Iowa, United States.
Soleilhavoup, C., Tsikis, G., Labas, V., Harichaux, G., Kohnke, P.L., Dacheux, J.L., Guérin, Y., Gatti, J.L., de Graaf, S.P., Druart, X., 2014. Ram seminal plasma proteome and its impact on liquid preservation of spermatozoa. J. Proteomics 109, 245–260.
Talevi, R., Gualtieri, R., 2010. Molecules involved in sperm-oviduct adhesion and release. Theriogenology 37, 796–801.
Utt, M.D., 2016. Prediction of bull fertility. Anim. Reprod. Sci. 169, 37–44.
Vicente-Fiel, S., Palacín, I., Santolaria, P., Fantova, E., Quintín-Casorrán, F.J., Sevilla-Mur, E., Yániz, J.L., 2014. *In vitro* assessment of sperm quality from rams of high and low field fertility. Anim. Reprod. Sci. 146, 15–20.
Villemure, M., Lazure, C., Manjunath, P., 2003. Isolation and characterization of gelatin-binding proteins from goat seminal plasma. Reprod. Biol. Endocrinol. 1, 39–50.
Wheaton, J.E., Windels, H.F., Johnston, L.J., 1992. Accelerated lambing using exogenous progesterone and the ram effect. J. Anim. Sci. 70, 2628–2635.
World Health Organization, 1992. WHO Laboratory Manual for the Examination of Human Semen and Sperm-cervical Mucus Interaction, 3rd edition. Cambridge University Press, Cambridge, pp. 11–13.
Yue, W., Shi, L., Bai, Z., Ren, Y., Zhao, Y., 2009. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of ram seminal plasma proteins and their correlation with semen characteristics. Anim. Reprod. Sci. 116, 386–391.
Zou, C., Yang, Z., 2000. Evaluation of sperm quality of freshly ejaculated boar semen during *in vitro* storage under different temperatures. Theriogenology 53, 1477–1488.