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Seminal Plasma Proteins as Potential Markers of Semen Characteristics in Buffalo and Bovine Bulls

Kadoom, A.K. ; F. E. El-Keraby ; A.F. Ashour and E. M. El-Siefy
Animal Production Res. Inst., Agric. Res. Center, Ministry of Agric., Dokki, Egypt

ABSTRACT

This study aimed to find a link between seminal plasma protein profile and semen characteristics, freezing ability and subsequent field fertility of bovine and buffalo bulls. Pre- and post-thawed semen of five Holstein and four buffalo bulls was used in this study. Total pattern of proteins precipitated from seminal plasma of bovine and buffalo bulls showed four different proteins of apparent molecular masses, 14 kDa, 15 kDa and 30 kDa with informative polymorphism in number and intensity of bands in both buffalo and cattle seminal plasma proteins. Seminal proteins bands (BSA-A1, A2, A3 and BSA-30) were positively correlated with percentages of motility and livability of spermatozoa, while negatively correlated with percentage of sperm abnormalities and acrosomal integrity in pre- and post-thawed semen of buffalo and bovine bulls. . It could be concluded that seminal plasma proteins can be used as a predicting measure for quality of bovine and buffalo semen. Further studies might be needed for quantitative determination of seminal plasma proteins which might give a better indicator for bull fertility when compared to the descriptive methods.

Keywords*:* Bovin, buffalo, semen, freezing, seminal proteins, bands, fertility prediction.

INTRODUCTION

Physical semen characteristics and sperm morphology measurements are not always indicative of bull fertility. Accurate and predictive protein markers are still needed for evaluation of inherent bull fertility (Foxcroft et al., 2008). Seminal plasma and sperm proteins have been reported as markers of fertility in different species. High fertile bulls have higher expression of seminal plasma proteins which might be involved in energy metabolism, cell communication, spermatogenesis, and cell motility (Peddinti, 2008). These proteins are products secreted from the seminal vesicles and ampullae, and their biochemical characteristics have been well-described (Moura et al., 2006 a and b).

Potential markers of fertility with several molecules are present in the seminal plasma milieu (Arlindo and Moura, 2005). *In vitro* testing of proteins purified from male and female reproductive fluids demonstrated that they facilitated sperm capacitation, fertilization and early embryo development (Killian, 2012) by stabilizing the sperm against premature capacitation and spontaneous acrosome reaction in addition to protection of the sperm from freezing-induced damage (Moura et al., 2006a).

Certain bovine seminal proteins are reported to induce an important cholesterol efflux from epididymal spermatozoa during capacitation (Therien *et al.,* 1998), while others (oviduct binder sperm proteins) have been considered as a key in establishing the sperm population in the oviduct isthmus (Gwathmey *et al.,* 2006). The prevalent protein in seminal plasma of higher-fertile bovine bulls is the 55 KDa proteins.

The biological properties of bovine seminal plasma (BSP) proteins have been extensively studied (Manjunath and Therien, 2002). It has been reported that BSP reverts the cold-shock damage on ram sperm membrane (Jobim *et al.,* 2004). BSP contains a family of major proteins, designated BSP-A1/-A2 and BSP-A3, with apparent molecular masses, ranging from 15 to 17 kDa, and the BSP-30 kDa protein with molecular mass of 28–30 kDa, BSP make up 40–50% of the total proteins in domestic bulls.

Aim of the current study was to found a link between seminal proteins and sperm characteristics, freezing ability and field fertility of bovine and buffalo bulls as prediction tool for fertility of bovine and buffalo bulls.

MATERIALS AND METHODS:

This study was carried out at the International Livestock Management Training Center (ILMTC), Sakha, belonging to the Animal Production Research Institute, Ministry of Agriculture.

Animals*:*

Five Holstein and four buffalo bulls were used in this study. All bulls were healthy and clinically free of external and internal parasites. Palpation of the external genitalia of these bulls showed that they were also typically normal. The testicular tone was glandular, almost similar in size and moved freely up and down within the scrotal pouches.

Semen collection and evaluation:

Bulls were sexually prepared by teaser bull and semen was collected using artificial vagina. Two frequent ejaculates at the same day were collected twice weekly from each bull. Immediately after collection, ejaculates were measured (semen volume) and held in a water bath at 35-37^{o}C. Ten and eight ejaculates were collected from each buffalo and bovine bull, respectively. Semen was evaluated pre- and post-freezing for motility, livability, abnormality and acrosome status.

Semen was centrifuged directly for seminal plasma separation. Semen was diluted at a rate of 20 x 10^6 life sperm per straw with Tris-egg yolk extender (3.025 g Tris; hydroxymethyl amino methane, 1.675 g citric acid, 0.75 g glucose, 15 ml egg yolk, 7 ml glycerol, 0.005 g streptomycin, and 0.25 mg linco-spectin) and completed with double distilled water up to 100 ml. The Tris-egg yolk extender was gently mixed and warmed up to 37^{o}C in a water bath during semen extension. The extension of ejaculates with tris-egg yolk was gradual for further processing and freezing. Vials

containing the extended semen were placed in a water bath at 37°C and cooled gradually in a refrigerator at 5°C for 4 hours as an equilibration period.

At the end of equilibration period, the extended semen was loaded in 0.25 ml labeled French straws containing about 20 x10^6 motile sperm using a semen filling machine fixed in cooling cabinet at 5°C. Straws were transferred into a processing canister and located horizontally on static nitrogen vapor 4 cm above the surface of liquid nitrogen for 10 minutes. Straws were then placed vertically in a metal canister and immersed completely in liquid nitrogen container for storage at –196°C.

For semen thawing, straws were dipped into a water bath at 35°C for 30 seconds. The percentage of live sperm was carried out according to Hancock (1951), while assessment of the percentage of intact acrosome was conducted as indicated by Watson (1975).

Quantitative and qualitative estimation of seminal plasma proteins:

Extraction of seminal plasma proteins:

Protein profile comparison was performed using Sodium Dodocyle Sulphat (SDS-PAGE). All the chemicals used for SDS-PAGE were purchased (UKLtd. Chalfont Bucking Hampshire, UK).

Then, frozen seminal plasma samples were thawed, centrifuged at 10,000 g for 60 min at 41ºC. 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 ºC with four volumes of ice-cold acetone and centrifuged for 15 min at 8000 *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.

Determination of protein molecular weight (SDS-PAGE) by electrophoresis:

Nine frozen seminal plasma samples (5 samples from Holstein bulls and 4 from buffalo bulls) were thawed at room temperature. For analytical gels, each of the seminal plasma samples was adjusted to 200 µg of total protein in 100 µl of rehydration buffer solution [7M urea, 2M thiourea, 20 mM DTT, 0.5% (v/v) IPG buffer at pH3–10]. Next, the mixture was cup-loaded on to their SDS-PAGE in 15% polyacrylamide. Samples were concentrated at 70 V for 10 min; separation was performed at 120 V for 4 h. Gels were stained with Coomassie Brilliant Blue, G-250 (Asadpour, 2012).

Density of the seminal protein bands are transformed into numerical score as follow: 0= not clear, 1= light color band, 2= medium color band, 3= high color band and 4= very high color band for statistical analysis.

Statistical analysis:

Statistical analysis was carried out using the Statistical Package for Social Sciences programme (SPSS), version 15.00, software for windows (SPSS Inc. Chicago, IL, USA). Statistical analysis was performed after arcsine transforming of the percentage values. The significant differences were set at P<0.05 level using Duncan Multiple Range Test (Duncan, 1955).

RESULTS AND DISCUSSION

Effect of freezing on postthawed semen parameters:

Buffalo semen:

Semen characteristics of buffalo bulls before freezing are shown in Table 1. Results showed that ejaculate volume averaged 3.2±0.19 ml, while percentages of motility livability, abnormality and intact acrosome of spermatozoa were 58.7±3.13, 60.5±2.94, 16.7±2.05 and 95.0±0.50%, respectively. Significant (P<0.05) individual differences were detected with respect to all physical sperm characteristics in pre- and post-thawed semen. Similar results due to freezing-thawing were previously reported by Mahmoud *et al.* (2013), who showed that variation between bulls were clear in post-thawing semen motility and live sperm percentage. They added that fresh buffalo semen volume and sperm concentration varied significantly (P<0.05) between bulls, while sperm motility and livability percentages were not affected in post-thawed semen.

Percentage of sperm with intact acrosomes averaged 95.0±0.50 and 88.8±1.26% in fresh and frozen semen. Freezing of bad quality semen significantly decreased of both life sperm count, their advanced motility and the intactness of acrosome. These results are in agreement with Kumar *et al.,* (2014), who mentioned that intactness of sperm acrosome of fertile bulls was significantly higher than sub-fertile bulls. Additionally, Singh *et al.* (2014) found that sperm motility had a high significant correlation with first service of conception.

Table 1. Semen characteristics of pre- (fresh) and post-thawed buffalo semen.

Semen parameters	R	198		219		13490		18685		Overall mean	
		Fresh	PF	Fresh	PT	Fresh	PT	Fresh	PT	Fresh	PT
Volume(ml)	10	2.6± 0.25^{b}	-	3.9± 0.50^{a}	-	3.5± .17^{a}	-	2.8± 0.38^{b}	-	3.2± 0.19	-
Motility %	10	66.0± 3.23^{b}	35.5± 1.38	67.0± 3.09^{b}	36.5± 1.98	74.0± 1.45^{a}	38.0± .82	28.0± 1.70^{c}	10.5± 1.38*	58.7± 3.13	30.1± 1.95
Life sperms %	10	68.8± 2.30^{a}	31.1± 1.24	69.0± 1.28^{a}	32.3± 1.42	72.1± 2.46^{a}	34.6± .50	32.2± 4.14^{b}	7.2± 1.41*	60.5± 2.94	26.3± 1.87
Abnormalities %	10	13.8± 2.91^{b}	13.9± .28	15.2± 3.66^{b}	14.7± .40	7.9± 1.34^{b}	15.6± .40	29.8± 4.47^{a}	25.8± 3.04*	16.7± 2.05	17.5± 1.07
Intact acrosome %	10	92.5± 0.42^{b}	95.3± .15	96.2± .42^{a}	76.4± 1.54*	94.5± 1.29^{a}	91.5 ±.76	96.9± 0.46^{a}	92.0± .82	95.0± .50	88.8 ±1.26

Means denoted within the same row for pre- or post-thawed semen with different superscripts are significantly different at P<0.05. * Significant difference between pre- and post-thawed semen for each bull. Number of replicates for each bull was 10 ejaculates.

Bovine semen:

Table (2) for bovine semen showed that semen volume averaged 4.4±0.19 ml, while percentages of motility, livability, abnormality and intact acrosome of spermatozoa were 66.8±2.16, 71.0±1.94, 13.8± 1.39 and 92.2±0.96%, respectively. The differences among bulls

was significant (P<0.05) in fresh semen. These results agreed with that of Ray and Ghosh (2013), who noticed that bulls varied significantly (P<0.05) in all their ejaculate characteristics. Moreover, hypo-osmotic swelling (HOS) test significantly differed (P<0.01) and positively correlated with percentage of progressive motile and live sperm.

Marked reduction in all semen characteristics were observed in post-thawed as compared to fresh semen. Chowdhury *et al.* (2014) found that the overall values of post-thawing intact acrosome sperms were 73.7±0.31% with no significant difference among the cattle breeds, while Ray and Ghosh (2013) noticed a negative correlation between HOS test and both abnormal sperm and intact acrosome.

Table 2. Semen characteristics in pre- (fresh) and post-thawed bovine semen.

Semen parameters	R	536		604		1502		1505		6690		Overall mean	
		Fresh	PT	Fresh	PT	Fresh	PT	Fresh	PT	Fresh	PT	Fresh	PT
Volume (ml)	8	3.8± 0.09^{a}	-	5.5± 0.19^{c}	-	3.5± 0.50^{a}	-	4.7± 0.44^{b}	-	4.5± 0.33^{b}	-	4.4± 0.19	-
Motility %	8	76.9± 2.10^{b}	39.3±1. 48*	75.0± 2.50^{b}	45.63± 1.48**	55.6± 4.06^{a}	18.1± 0.92	51.2± 3.63^{a}	23.1± 0.92	75.6± 1.48^{b}	40.0± 1.64*	66. 8± 2.16	33.2± 1.80
Life sperms %	8	77.8± 2.81^{b}	35.7±1. 19*	78.1± 2.33^{b}	40.88± 1.82**	61.7± 5.46^{a}	15.7± 0.82	60.2± 2.67^{a}	19.25± 0.68	77.1± 2.56^{b}	34.0± 2.07*	71.0± 1.94	29.1± 1.68
Abnormalities %	8	8.4± 1.44^{a}	14.7±0. 68*	10.8± 2.48^{b}	11.75± 0.59	18.1± 3.17^{c}	24.6± 1.10*	19.7± 4.12^{c}	21.63± 1.00*	12.2± 2.22^{b}	15.3± 0.82*	13.8± 1.39	17.6± 0.84
Intact acrosome %	8	95.6± 1.48^{b}	81.8±2. 49*	92.5± 2.32^{a}	68.63± 2.56	94.3± 1.48^{b}	88.1± 0.92**	86.8± 2.92^{a}	88.50± 1.03**	91.8± 0.92^{a}	87.2± 2.02**	92.2± 0.96	82.8± 1.46

Means denoted within the same row for pre- or post-thawed semen with different superscripts are significantly different at P<0.05. * Significant difference between pre- and post-thawed semen for each bull. Number of replicates for each bull was 8 ejaculates.

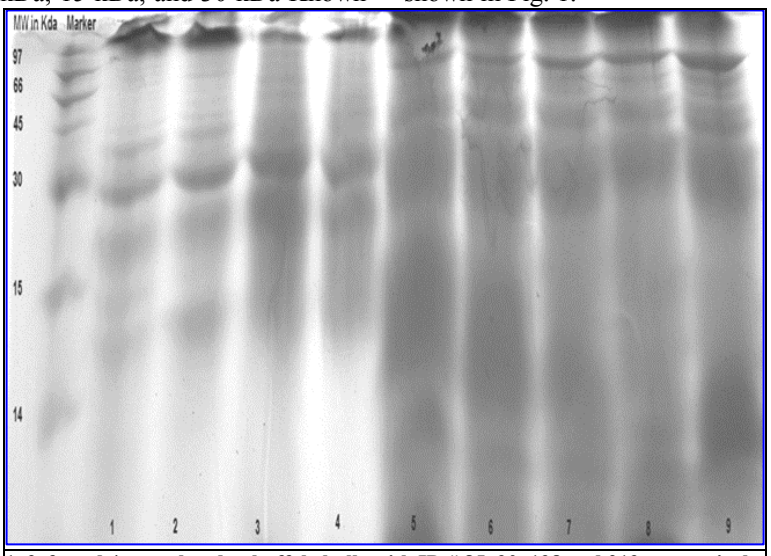
Expression of seminal plasma proteins

The total pattern of proteins precipitated from bovine and buffalo seminal plasma was electrophoresed on SDS-PAGE fraction. The electrophoretic patterns revealed informative polymorphism in the number and intensity of bands concerning both species. Banding patterns were analyzed by SDS-PAGE (Fig. 1), which revealed the presence of four different proteins with apparent molecular masses of 14 kDa, 15 kDa, and 30 kDa for buffalo and bovine seminal plasma proteins. Two bands, BSP-A1-A2 related to 14 and 15 KDa and BAS-A3 related to 30 KDa were observed, respectively (Fig.1).

Expression of seminal plasma proteins in the buffalo bull:

Seminal plasma protein bands expressed in the buffalo bull showed different proteins of apparent molecular masses, 14 kDa, 15 kDa, and 30 kDa Known collectively as BSA-A1/A2 and BSA-A3. The expression of BSA-A1/A2 and BSA-A3 were very high (very dark bands) in bull No. 198 and 219. While BSA-A1/A 2, showed dark (high expression) in bull No. 90 and 85. Otherwise, BSA-A3 protein showed high expression (dark band) in bull No. 90, however, its expression was faint (low expression) in bull No. 85 as shown in Figure (1).

Expression of seminal plasma proteins in the bovine bull*:* Concerning the expression of bovine seminal plasma proteins, the BSA-A1/A2 was very dark (very high gene expression) in bull No.90 and bull No.536, while its expression in bull N0. 604 was dark (high expression) and it was absent in bull No. 1502 and 1505. However, BSA-30 band was very dark (very high expression) of bull No. 90, 536, 604 and 1505, but it was very dark (high expression) in bull No. 1502 as shown in Fig. 1.



1, 2, 3, and 4 are related to buffalo bulls with ID # 85, 90, 198 and 219, respectively. While, 5 , 6, 7, 8 and 9 are related to bovine bulls with ID # 90, 536, 604, 1502 and 1505, respectively.

Fig. 1: Electrophoretic pattern of seminal plasma proteins of bovine and buffalo bulls.

Correlation between sperm parameters and seminal plasma protein expression:

Expression density of seminal plasma protein bands (BSA-A1, A2, A3 and BSA-30) was positively correlated with sperm motility and livability in both fresh and frozen-thawed semen of buffalo and bovine bulls (Killian *et al.,* 1993; Bellin *et al.,* 1998). In fresh semen, a highly significant negative correlation (P>0.01) between expression of BSA-A30 with sperm abnormalities and the integrity of the acrosome in buffalo bull is shown in Table (1). Based on protein expression, Chacur, (2012) noticed that bands for BAS-A1-A2 and BAS-A3 were related to low fertile bull, where bands of BSA-30 KDa was related to high fertility, which could have strong inhibitory effects on gene expression in sensitive buffalo bull genotypes. Those particular bands could be considered as a positive biochemical marker associated with fertility in the buffalo bull. While, in fresh bovine semen, negative significant correlations were found between sperm abnormalities and expression of BSA-A1 and BSA-A BSA-A2 (P>0.01). These results are agreeable with the previous results of Veronic and Puttaswany (2000), where they indicated that the BSA-A1, BSA-A2, BSA-A3 and BSA-30 KDa proteins expression were enhanced in fertile bovine seminal plasma. Moreover, post-thaw semen characteristics were significantly correlated (positively in case of motility and livability and negatively in case of sperm abnormalities and acrosomal integrity) with the expression of seminal plasma proteins (Table 3). In agreement with our results Sharma et al. (2012) found highly significant (P<0.01) correlation between various semen evaluation parameters except for the gross morphological abnormalities. However, a significant (P<0.05) negative correlation was found between the acrosome integrity and gross morphological abnormalities as reported by Chowdhury *et al.* (2014).

In contrast to the present results of the current study, Veronica and Puttaswamy (2000) noticed that protein expression did not differ (P>0.05) with fertility grouping, and the majority of the functional proteins were highly expressed in seminal plasma of non-cryopreserved semen, while the cryopreserved semen contained mainly structural/extender derived proteins. In this espect, 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). 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 (Rodríguez Martínez *et al.,* 2011). Moreover, the cryopreservation extender used may have different effects on the loss of sperm surface proteins after even brief storage periods in liquid nitrogen (Lessard et al., 2000). Screening for presence of seminal plasma proteins can be included in the regular breeding soundness examination for selection of bulls (Karunakaran and Devanathan, 2016).

Table 3: Correlation between seminal proteins and different semen parameters in both buffalo and bovine bull semen

Semen parameter	Pearson Correlation of semen parameters							
	Buffalo bulls				Bovine bulls			
	BSA-A1	BSA-A02	BSA-A3	BSA-30	BSA-A1	BSA-A2	BSA-A3	BSA-30
Volume, ml	0.04	0. 4	0.16	0.04	0.10	0.10	0.24	0.39*
Sperm Motility, %	0.40*	0.40*	0.81**	0.40*	0.80**	0.80**	0.33*	0.42**
Livability, %	0.46**	0.46**	0.82**	0.46**	0.66**	0.66**	0.29	0.38*
Sperm abnormality, %	-0.17	-0.17	-0.49**	-0.17	-0.47**	-0.47**	-0.19	-0.25
Intact acrosome, %	-0.24	-0.24	-0.37*	-0.24	0.23	0.23	-0.20	-0.18
Post-thaw motility, %	0.48**	0.48**	0.86**	0.48**	0.86**	0.86**	0.51**	0.68**
Post-thaw livability, %	0.46**	0.46**	0.86**	0.46**	0.84**	0.84**	0.46**	0.64**
Post-thaw sperm abnormality, %	-0.48**	-0.48**	-0.70**	-0.48**	-0.79**	-0.79**	-0.49**	-0.67**
Post-thaw intact acrosome, %	-0.38*	-0.38*	-0.32*	-0.38*	-0.35*	-0.35*	-0.03	-0.30

***Correlation coefficients (r) are significantly different at P<0.05.**
**** Correlation coefficients (r) are significantly different at P<0.01.**

CONCLUSION

It could be concluded that seminal plasma proteins can be used as potential markers of sperm freazability and fertility of bovin and buffalo bulls. Further studies are needed for quantitative determination of seminal plasma proteins and fertility of bovine and buffalo bulls which might be better than the qualitative method.

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بروتينات بلازما السائل المنوى كواسمات نسبية ذات الصله بخصائص السائل المنوى فى الابقار والجاموس"

عبدالجواد خليفة قادوم , فكرى السيد القربى , أيمن فؤاد عبد الهادي عاشور و الشناوى محمد الصيفى

معهد بحوث الإنتاج الحيواني - مركز البحوث الزراعية - وزارة الزراعة

تهدف هذه الدراسة على الوصول إلى رابط بين بروتينات وخصائص السائل المنوى لكل من الطلائق الجاموسى والبقرى , وقد تم إستخدام خمس طلائق بقرى و أربع جاموسى لجمع قذفات السائل المنوى من كل من هذه الطلائق (١٠ قذفات من الجاموسى و ٨ من البقرى) على التوالى لتقييم السائل المنوى وتجهيزه وتجميده وفصل بروتينات السائل المنوى (BSA-(A1, A2, A3& BSA-30. وقد اظهرت النتائج أن الفصل الكهربى لهذه ابروتينات وجود حزم متباعدة ومختلفة فى الوزن الجزيئى والكثافة (14 kDa, 15 kDa, and 30 kDa) بالإضافة إلى وجود ارتباط معنوى طردى بين هذه البروتينات مع حركة وعدد الحيوانات الحية سواء قبل او بعد التجميد وبشكل عكسى مع تشوهات الحيوانات المنوية وتماسك الأكروسوم للحيوان المنوى فى كل من الطلائق الجاموسى والبقرى. ومن هنا نستخلص ان تقدير بروتينات السائل المنوى بشكل كمى قد تكون مفيدة فى التقييم المستقبلى للطلائق والتنبؤ بخصوبتها المستقبلية. كما نوصى بإجراء المزيد من الدراسات على التتقدير الكمى لهذه البروتينات والتى قد تفيد فى تقييم السائل المنوى مستقبلا .