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Analysis of Seminal Plasma Proteins of South Indian Jersey and Hybrid Bulls and their Correlation with Semen Quality

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ABSTRACT

This is a preliminary study of the protein profiles of the semen in Jersey breed bulls. Jersey bulls were mainly used for fertility purposes; they were grouped based on the fertility, such as highly fertile group (95% fertility), medium fertile group (65-70%), low fertile group (45-50%) and hybrid bulls. Semen of 20 Jersey and hybrid bulls was collected through artificial vagina. The semen characteristics includes volume, pH value, viscosity, viability, sperm concentration, agglutination, total motility and their group, sperm morphology and Hypo-Osmotic Swelling test (HOS) were carried out immediately after collection. Total antioxidant capacity test by using catalase and cholesterol analysis were also carried out. Quantification of the amino acids and proteins were carried out by Ninhydrin and Lowry method, protein analysis by Sodium Dodecyl Sulphate (SDS) was carried out for the following categories. (1) Highly fertile group (Jersey), (2) Medium fertile group (Jersey), (3) Low fertile group (Jersey) and (4) Hybrid group. Analysis of semen protein reveals about seventeen protein bands of sizes ranging between 14 and 205 kDa were identified. Among those proteins bands, the relative protein content of nine bands shows significantly different from each group. The rest of the proteins bands seem to have some positive and/or negative correlation on the semen characterizes or fertility. The antioxidant capacity of highly fertile and hybrid group was very high compare to low fertile group of Jersey. This study clearly indicates the bull seminal plasma proteins are associated with fertility and as well as determination of semen quality and ultimately decides the fertility.

Key words: Semen, seminal plasma, jersey bulls, catalase, antioxidants, ninhydrin test

INTRODUCTION

Semen consists of mature spermatozoon and swims in a highly complex features seminal fluid. Seminal fluid contains a complex mixture of organic compounds secreted from epididymis, testes and other accessory sex glands, influences sperm morphology, motility, sperm concentration acrosome reaction and fertility (Mann and Lutwak-Mann, 1981). Biochemical analysis of seminal fluid reveals that it is composed of amino acids, proteins and polypeptides. These molecules of seminal plasma determines semen quality and fertility were different in different species, some are reported to be associated with the fertility. Good quality semen is the most important factor to implement breeding programs (Stradaioli *et al*., 2004).

The presence of proteins, lipids and hormones in seminal plasma suggests that a large part of the physiological functions of this fluid are not yet ascertained. It is a fact that components especially, high levels of fructose and zinc are not present in other body fluids. Effective semen

storage is important for effective genetic improvement and utilization of the seminal plasma to understand the complexity of seminal plasma (Wusiman *et al*., 2012). The functions of the seminal plasma were large and that includes coagulation of the semen soon after ejaculation, the buffering property of this in neutralizing the acidic nature of vagina (Mann and Lutwak-Mann, 1981).

In Assisted Reproductive Technology (ART) the seminal plasma is routinely diluted or removed during the processing of semen for cryopreservation and this can be either positive or negative association with fertility (Maxwell *et al*., 2007). The effects of vitamins and on reproductive parameters have been assessed in human and boars (Adabi *et al*., 2008). The seminal vesicle secretion accumulates in the lumen of the reproductive gland after the puberty. This fluid constitutes a portion of the seminal plasma protein on ejaculation and that influences the metabolism, motility and even morphology (Manco and Abrescia, 1988; Metafora *et al*., 1989; Peitz, 1988). The adsorption of large quantity of seminal plasma proteins has been studied by Metz *et al.* (1990). It is generally consider that excessive dilution of the seminal plasma during processing is detrimental to sperm function (Mann, 1954; Wusiman *et al*., 2012). The effects of seminal plasma on pre-freeze and post-thaw spermatozoa motility of epididymal spermatozoa have been investigated but the results were contradictory. To protect from the ROS-mediated damage, sperm and the seminal plasma having the antioxidant system composed of enzymatic and non-enzymatic molecules. The free radical scavenging activity of catalase, preserving its integrity and hence maintain the normal sperm. Aziz *et al*. (2004) studied the role of antioxidants in protecting the nuclear and mitochondrial genome from base modifications.

BSP-like proteins have been extensively studied in other species. In stallion, HSP-1 (72 kDa, pI 5.6) was proved positively correlated with fertility and HSP-2, HSP-3 and HSP-4 were negatively correlated with fertility (Calvete *et al*., 1995; Brandon *et al*., 1999). However, the correlation between the seminal plasma proteins and its semen characteristics has not been investigated in bulls. The present study was performed to investigate the fertility-associated proteins in bull seminal plasma and the correlation between specific proteins and semen characteristics in bulls.

MATERIALS AND METHODS

This study used all pure adult South Indian bull were proved healthy. They were fed according to the standard conditions. The bulls for the control group were selected from the bulls with an average fertility rate of between 70-75%. High fertility group, the bulls with above 95% fertility rate and low fertility rate of less than 45% were analyzed. The medium fertile group (65-70%) was also analyzed.

Sample collection: Semen samples of Jersey and Hybrid South Indian bulls were collected during January to March 2011 from the farms in and around Vellore. Fresh semen samples were collected in the farm at a pre-warmed condition (42-45°C) through artificial vagina. On each collection, two ejaculates were taken at an interval of between 15-30 min between successive ejaculates and each ejaculate was preceded by a period of sexual preparation consisting of at least two false mounts separated by about a 1 min restraint. During this collection the first ejaculate is discarded, usually it consists of fluid, which is whitish milky in color. Then second ejaculate is collected in the same manner which is then used for the molecular analysis (Calvete *et al*., 1995; Brandon *et al*., 1999).

Evaluation of semen: The ejaculates were collected thrice in a week with the aid of an artificial vagina at the time between 6:00-7:00 AM. Semen collection was done for 3 weeks consecutively.

The replicates for a single animal were six. Semen analysis and evaluation was done immediately after collection. The ejaculated semen volume and the pH were determined. Sperm motility and their group were determined by using microscope. A drop of semen was placed on the pre-warmed slide (37ºC) and covered with cover slip. The motile spermatozoa in percentage were observed and classified their group using phase-contrast microscope at 100X magnification. To determine the live spermatozoa count, the droplet was stained with eosin-nigrosin and counted by using phase-contrast microscope under 40X magnification. To determine the sperm concentration, semen was diluted by using phosphate buffer at pH 7.0 at the ratio of 1:20 and then a drop was placed on Makler's counting chamber. The numbers of cells were counted by using phase-contrast microscope under 40X magnification (Metz *et al*., 1990).

Preparation of seminal plasma: The plasma was separated immediately after collection from the total ejaculate. Fresh semen was centrifuged at 8000 rpm for 10 min at 4ºC. The supernatant was transferred into centrifuge tubes of 2 mL and then centrifuged at 12000 rpm for 20 min at 4ºC to eliminate the remaining sperm. The seminal plasma was kept at -20ºC until used after the total protein concentration was determined by using the spectrophotometer at 280 nm. For the preserved samples, the straw is cut down at the sealed side and it is punched by the smart gun opposite to the sealed side. The sample is collected in the micro centrifuge tube. Immediately after collection, the sample is centrifuged at 10000 rpm for 5 min. The cells are pelleted and the supernatant is discarded which consists of debris and the sodium citrate, egg yolk, whatever material present in the sample except the cells, then the cells were stored at -20°C (Stradaioli *et al*., 2004).

Protein and amino acid estimation: Protein was determined as explained by Lowry method. Amino acids were determined by standard ninhydrin method. The standard amino acids were prepared. The volume was making up to 1 mL by 1 N acetate buffer. To the 1 µL of sample, 900 µL of buffer was added, 1 mL of ninhydrin reagent was then added to all the test tubes including the standards. The tubes were kept for boiling for 15 min 2 mL of diluents (50% ethanol) was added to all the test tubes. The absorbance was read at 570 nm. The concentration of amino acids was determined (Wusiman *et al*., 2012).

Analysis of cholesterol: Free cholesterol present in the seminal fluid was analysed and determined by chromatography on Silica gel 60-250 TLC (0.25 mm) sheets. The developing solvent used here was n-hexane/ethyl ether/acetic acid (38:12:1, v/v). After the plates were spotted with the samples, air-dried and immersed in a staining solution of 0.05% Brilliant blue-R in 20% methanol (v/v) for 1 h. The plates were then de-stained with 20% methanol for 30 min. It was air-dried successively for 15 min quantitative assessment was done by comparing with the reference standards. No cholesterol esters were able to found in the samples analysed in this paper (Stradaioli *et al*., 2004).

Catalase assay for antioxidant: Catalase was estimated by the standard protocol. Hydrogen peroxide will be degraded by catalase and which can be measured directly by decreasing in the absorbance at 240 nm. Five hundred microliter of 50 mM phosphate buffer (pH 7.0) and 500 µL of 30 mM hydrogen peroxide were taken in a cuvette, 30 µL of seminal plasma was added to this and mixed well. The decomposition of hydrogen peroxide was followed by decrease in extinction at 240 nm which was recorded at each 30 sec for 5 min (Stradaioli *et al*., 2004). The difference in

extinction per unit time is the measure of catalase activity. One unit of catalase activity was defined as the amount of catalase which decomposes 1 μM of hydrogen peroxide in 1 min. The catalase activity was then calculated from the change in absorbance and it was expressed as Enzymes (U μL^{-1}).

Sample processing for SDS PAGE: The stored samples were taken and processed for protein analysis by SDS PAGE. Equal amount of gel loading dye (2.5 mL of 4X Tris Cl. - SDS at pH 6.8, 2 mL of Glycerol, 0.4 g of SDS, 200 μL of 2-mercaptoethanol, 0.1 g of bromophenol blue, dissolved in 10 mL of Milli Q water) was added, mixed and boiled at100°C hot water bath for 10 min. Then the SDS-PAGE gel was prepared. Separating gel was prepared by mixing 2.5 mL of 30% Acrylamide and N, N-methyl bis- acrylamide, 1.8 mL of 4X Tris Cl at pH 8.8, 3.05 mL of Milli Q water, 80 μL of freshly prepared APS and 40 μL of TEMED. Mix it well and poured in PAGE plates. It was allowed to polymerize for 30 min. Stacking gel was prepared by mixing 0.65 mL of 30% Acrylamide and N, N-methyl bis-acrylamide, 1.25 mL of 4X Tris Cl-SDS buffer at pH 6.8, 3.05 mL of Milli Q water, 40 μL of freshly prepared APS and 25 μL of TEMED. It was poured above the separating gel and allows it to polymerize 20 min. The comb was inserted into the SDS plates without disturbing the gel volatility. This set up was allowed for 1 h. The set up was made ready to load the sample, after loading allow the gel to run in the buffer for 3 h or until it reaches the bottom. The molecular weight was estimated by using the protein low-molecular as well as high-molecular weight protein marker. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 (0.15%) including 50% methanol and 10% acetic acid and de-stained in a mixture of 25% methanol, 10% acetic acid and distilled water until no background was detectable (Stradaioli *et al*., 2004).

Data analysis: The gel was analyzed to determine the molecular weight of the protein. The values were expressed in terms of Mean±Standard error of mean. Tukey's test was used subsequently for comparing the mean values at the level of $p \leq 0.05$. The correlation of the relative protein content with different semen parameters were examined by using Pearson's coefficient test.

RESULTS AND DISCUSSIONS

The semen evaluation was done for control group and all the other four groups were shown in Table 1. Semen quality parameters including pH, concentration, viability, morphology in the low fertility group of Jersey were significantly lower ($p<0.05$) than the higher fertility and medium fertility group of Jersey and hybrid group. The antioxidant capacity by catalase assay was done for all groups in the study conducted by Stradaioli *et al*. (2004) also shows catalase activity positively correlated with pH and sperm concentration. The activity was found to be 0.0086 Enzyme U μL^{-1} for control groups with p-value of 0.026. For the highly fertile group it was 0.0088 Enzyme U μL^{-1} with p-values 0.023. For the medium and low fertile group it was 0.0070 and 0.0061 Enzyme U μL^{-1}, respectively with p-value of 0.067 and 0.0187. This result is also positively correlating with the results of Yue *et al*. (2009).

The molecular weight of proteins of low, medium and high molecular weight proteins including size ranges from 6.5-205 kDa for 17 distinct protein bands identified by SDS PAGE gel which was shown in Fig. 1. None of the sample show to contains all the seventeen bands in equal quantity, this result is correlating with the result of Manco and Abrescia (1988). Most of protein sample consists of fertility associated protein in identical proteins. The molecular

Table 1: Semen parameters evaluation for different groups based on fertility

Groups	Volume (mL)	pH	Sperm concentration (millions mL^{-1})	Total motility (%)	Active motile count (%)	Sluggish motile count (%)	Viability (%)	Morphologically abnormal (%)
Control	4.25±0.15	6.98±0.19	38.2±0.27	82.3±0.59	53.4±0.22	28.3±0.11	85.18±0.33	21.24±0.78
Low fertility (J)	3.87±0.22	7.29±0.22	32.19±1.28	61.2±0.64	32.12±0.69	31.12±0.18	64.34±0.56	31.69±0.23
Medium fertility (J)	4.12±0.21	6.91±0.24	40.12±0.98	73.3±0.44	40.13±0.34	35.35±0.76*	76.56±0.12	19.34±0.56*
High fertility (J)	4.29±0.23	7.22±0.21	43.10±0.94	85.12±1.98	73.26±1.24	15.69±0.19*	88.4±0.98	9.14±0.24
Hybrid (H)	4.98±0.28	7.12±0.14	47.15±0.11	81.17±0.86	73.12±0.76	9.12±0.56	83.45±0.45*	8.34±0.58

*Represents p<0.05 when compared to control, All the values were expressed with mean±SEM

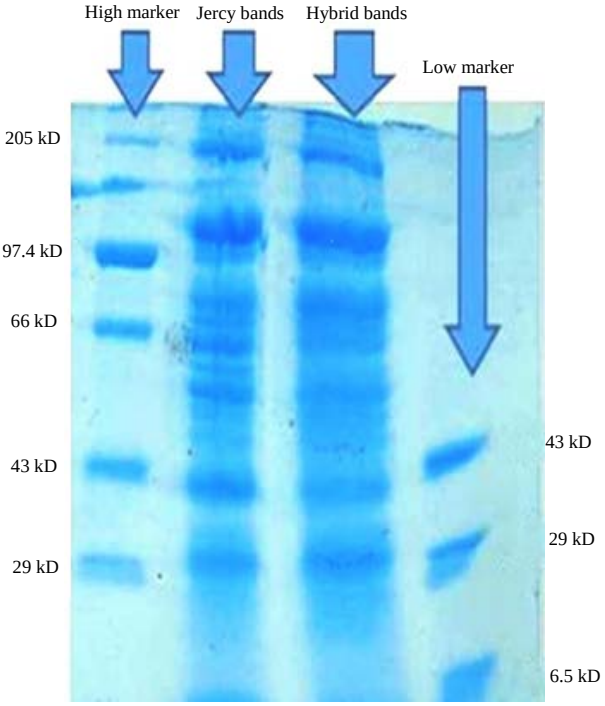

Fig. 1: SDS gel showing the bands of Jersey and hybrid bull, SDS PAGE was done for all the different fertility groups. 17 protein bands of molecular weight range from 6.5-205 KDa were observed. Based on the relative protein content the bands were correlated with the semen quality parameters

weight of proteins found with less than 40 was prominent in Fig. 1. The protein band of HMW 1 found with 205 kDa were not detected in some of the low fertility group of Jersey, but it was detected in the hybrid protein samples even with low fertile group which was shown in Fig. 1. These results were found to be similar to the results of Calvete *et al*. (1995) and Brandon *et al*. (1999). Four protein bands with molecular weight (from top, 97.4, 90.0, 29.1 and 14.3) were negatively correlated with semen parameter pH. Protein band 1 was positively correlated with sperm motility, viability and morphology. The protein bands with molecular weight (97.4, 90.0, 66.0 and 22.0) their relative protein content was not significantly different between low fertility group of Jersey and hybrid but somehow they were correlated with semen parameters. Two proteins (22.0 and 14.3) were negatively correlated with sperm concentration but proteins (205, 97.4) were found to be positively correlated with sperm concentration. These results were found to be uncertain when compared to the results of Barrios *et al*. (2000). The protein spots

with a molecular weight from 15-20 kDa accounted for 41% of the relative intensity of the spots of the gel which was shown in Fig. 1. In another study using SDS-PAGE, 20 protein bands were found in ovine seminal plasma and the most prominent protein was below 70 kDa (Barrios *et al*., 2000). The study conducted by Yue *et al.* (2009), protein spots with a molecular weight from 15-20 kDa accounted for 41% of the relative intensity of the spots of the gel. Some authors observed that when the molecular weights range between 12.5 and 83.9 kDa, the protein spots <21 kDa had the highest relative intensity using the gradient gel (Cardozo *et al*., 2006). Available reports reveals that proteins below 45 kDa, but in our study it was less than 40 kDa. Our results mainly focusing on the 8 bands that are associated with fertility in south Indian Jersey and hybrid bulls. SDS-PAGE analysis of alcohol-precipitated ram seminal proteins indicated the presence of about 25 proteins with molecular masses from 14-120 kDa, a group of proteins with a molecular mass of 15-16 kDa and 22-24 kDa was more predominant (Bergeron *et al*., 2005). The relative content of protein for low fertility group and high fertility group of Jersey were found to be with significant different in that fertility associated proteins. Molecular weights ranged from 15-115 kDa and the most prominent spots were those <30 kDa (Jobim *et al.,* 2005). Much more the band with 205 kDa protein which was not detected in low fertile group of Jersey and in some of the medium fertile group of Jersey might be associated with fertility in bulls. The high relative content of protein in band with molecular weight 160 in low fertile group could explain its negative correlation with sperm viability and concentration. These results were positively correlating with the results of Metz *et al*. (1990). The content of this protein was increased significantly in the breeding season and correlated with viability. However, the content of 72.45 kDa protein in our result was correlated with sperm concentration. More importantly, the study conducted by Yue *et al.* (2009), indicates that the relative content of seminal plasma protein could be an essential index to evaluate ram fertility and semen quality. The protein with low pH value (which was foud almost in all bands) determines the survival of the spermatozoa. Previous data determines that the proteins present in seminal plasma of Indian bulls were acidic and somehow the bands in hybrid bulls were showing alkalinity. The catalase assay proves that the antioxidant capacity of highly fertile group is high rather than the low fertile group of Jersey. This may help in enhancing the fertility of bull.

CONCLUSION

This research summarizes that the seminal plasma proteins of South Indian bulls shown significant difference between Jersey (high, medium, low fertile group) and hybrid. This will helpful in enhancing the fertility of bulls for cross breading. The relative protein content of seminal plasma evaluates the fertility and the semen quality. Our further research will be focused on the identification of a protein marker fertility associated proteins for bull semen to enhance the fertility.

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