Short communication

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of ram seminal plasma proteins and their correlation with semen characteristics

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Abstract

The present study was conducted to investigate fertility-associated proteins in ram seminal plasma and the correlation between specific protein and semen characteristics in sheep. Thirty-eight German merino sheep clinically proven healthy were chosen and divided into three groups according to fertility. Ejaculates were collected by an artificial vagina and semen characteristics (volume, pH value, motility, viability and concentration) were recorded. Seminal plasma was harvested by centrifugation and then subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) analysis in parallel with molecular weight standards. Fifteen protein bands with different molecular weights, ranging from 15.13 to 116.20 kDa, were identified on the gel. The results showed that the relative content of eight protein bands was significantly different between the high-fertility group (H-group) and the low-fertility group (L-group). Although the remaining seven protein bands showed no fertility-associated changes in their relative content, some of them were negatively or positively correlated with some semen quality parameters (motility, viability, concentration or pH value). Thus, this study indicates that ram seminal plasma contains specific proteins that are associated with fertility and semen characteristics. Also, these proteins could be utilised in developing a reliable and simple method to determine the ram fertility or semen quality.

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1. Introduction

Seminal plasma, which is a complex mixture secreted from the testes, epididymis and accessory sex glands, can affect sperm morphology, motility, acrosome reaction and fertility (Mann and Lutwak-Mann, 1981). Despite its physiological significance, the molecular composition of the seminal plasma is complex and the various proteins or polypeptides present in seminal plasma are poorly understood.

In recent years, several seminal plasma proteins have been identified, isolated and characterised. Evidence suggests that the protein composition of seminal plasma is different among species and that some seminal plasma proteins are associated with fertility in various species. Bovine seminal plasma (BSP) contains a family of major proteins designated BSP-A1/A2 and BSP-A3 with molecular masses ranging from 15 to 16.5 kDa and BSP 30 kDa with an approximate molecular mass of 28–30 kDa (Manjunath and Sairam, 1987). These proteins, collectively called BSP proteins, bind to the sperm surface and modulate sperm functions (Manjunath and Therien, 2002). BSP-like proteins have been extensively studied in other species. In stallion, HSP-1 (72 kDa, pl 5.6) was proved positively correlated with fertility and HSP-2, HSP-3 and HSP-4 were negatively correlated (Calvete et al., 1995; Brandon et al., 1999). It is reported that goat seminal plasma contains a group of proteins (called GSP proteins) that are structurally related to the BSP proteins detected in bull, boar and stallion (Villemure et al., 2003). Homologous proteins have also been identified in boar (Sanz et al., 1993), ram (Jobim et al., 2005) and canine seminal plasma (de Souza et al., 2007). Previous studies are generally related to the comparisons of seminal plasma composition between males of different fertility or the isolation and characterisation of specific seminal proteins that could influence sperm capacitation and fertilisation. Several studies have reported the seasonal variation of ram seminal plasma proteins (Cardozo et al., 2006; Ghaoui et al., 2007) and the effect of seminal plasma proteins on cold-shock membrane damage of ram spermatozoa (Pérez-Pé et al., 2001). However, the correlation between specific seminal proteins and semen characteristics in rams with differing fertility has not hitherto been well studied.

The present study was performed to investigate the fertility-associated proteins in ram seminal plasma and the correlation between specific proteins and semen characteristics in sheep.

2. Materials and methods

2.1. Materials

All pure adult German merino sheep used in this study were proved healthy clinically and were fed and managed under similar standard conditions. Fertility data on each sheep provided by Youyu Breeding Sheep Center was based on breeding records for more than 3000 inseminations using diluted fresh semen. The experimental animals were chosen using the method previously described (Killian et al., 1993). The animals for the control group were selected from sheep with an average fertility rate (between 75% and 80%). The high-fertility group (H-group) contained sheep with a fertility rate above 90% and the low-fertility group (L-group) had fertility below 40%. The groups contained 14 high-fertility sheep and 9 low-fertility sheep. Fifteen sheep with moderate fertility were selected as the control group (C-group).

2.2. Semen evaluation

Ejaculates were collected twice weekly with the aid of an artificial vagina (07:00–08:00 am) and semen collection was performed for 4 consecutive weeks (eight replicates for a single animal). Semen analysis was performed immediately after collection. Semen volume was determined with a graduated plastic tube and its pH value was measured using a pH-meter. Sperm motility was determined subjectively as the percentage of total motile sperm by microscopic examination. A drop of semen was placed on a pre-warmed slide (37°C) and covered with a cover slip. The percentage of motile spermatozoa was observed with a phase-contrast microscope at 100× magnification. After staining with eosin–nigrosin (Swanson et al., 1951), 200 spermatozoa in five different fields were counted using a phase-contrast microscope at 400× magnification to determine the percentage of live spermatozoa. For sperm concentration, semen was diluted at the ratio of 1:20 in distilled water and a drop of mixture
was placed on a haemocytometer chamber. The numbers of cells were counted under a phase-contrast microscope at 200× magnification. Sperm concentration was calculated and expressed as 10^9 ml^{-1}.

2.3. Preparation of seminal plasma

The seminal plasma was separated immediately after collection. Fresh semen was centrifuged at 1500 × g for 15 min at 5°C. The supernatants were transferred into 1.5 ml eppend of tubes and re-centrifuged at 10,000 × g for 30 min at 5°C to eliminate the remaining sperm. After the total protein concentration was determined using a spectrophotometer (Nanodrop, ND-1000, USA) at Protein A280 (protein’s absorbance at 280 nm) module, the seminal plasma was stored at −70°C until used.

2.4. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed to determine the molecular weight and relative content of various seminal plasma proteins. Seminal plasma samples were subjected to SDS-PAGE according to the method previously described (Laemmli, 1970) using a 10% polyacrylamide gel. A volume of 4.5 μl seminal plasma protein samples was mixed with 0.1% bromophenol blue containing 5% SDS, 20% glycerol and 10% β-mercaptoethanol. The molecular weight was estimated using protein low-molecular weight standards. The standards were phosphorylase B (9 kDa), bovine albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30.0 kDa), trypsin inhibitor (20.1 kDa) and lysozyme (14 kDa).

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.

2.5. Image acquisition and data analysis

Gel images were analysed to determine molecular weight and relative protein content using the Quantity One computer program (version 4.6.5, Bio-Rad, USA). Data analysis was performed using SPSS software program (version 13.0 for Windows). All values were expressed as mean ± standard error of mean (S.E.M.). Analysis of variance (ANOVA) with Tukey’s test was used subsequently for comparison of mean values at a significance level of P < 0.05. The Pearson’s correlation coefficient test was applied to examine the correlation of relative protein content with the semen parameters. Correlations with P < 0.05 were considered significant.

3. Results

The results of the semen evaluation of the three different groups are shown in Table 1. Semen quality parameters in the L-group were significantly lower (P < 0.05) than those in the H- and C-groups.

As is shown in Table 2, a total of 15 protein bands were identified and their molecular weights ranged from 15.13 to 116.20 kDa. The proteins with a molecular weight below 26 were prominent. However, no individual protein sample contained all 15 bands. The 116.20 kDa (band 1) protein was not detected in most of the semen samples obtained from the sheep in the L-group.

The relative protein content (%) of eight proteins (1, 2, 4, 10 and 12–15) was significantly different between the H- and L-groups. Of these protein fractions, the protein content (%) of band 4 and 15 in

<table>
<thead>
<tr>
<th>Groups</th>
<th>Volume (ml)</th>
<th>pH</th>
<th>Motility (%)</th>
<th>Viability (%)</th>
<th>Concentration (×10^9 ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-group</td>
<td>1.22 ± 0.14^a</td>
<td>6.69 ± 0.27^a</td>
<td>79.3 ± 0.59^a</td>
<td>80.3 ± 1.22^a</td>
<td>3.87 ± 1.22^a</td>
</tr>
<tr>
<td>H-group</td>
<td>1.23 ± 0.22^a</td>
<td>6.70 ± 0.10^a</td>
<td>78.7 ± 0.62^a</td>
<td>81.0 ± 0.86^a</td>
<td>3.86 ± 1.07^a</td>
</tr>
<tr>
<td>L-group</td>
<td>0.87 ± 0.25^b</td>
<td>6.52 ± 0.19^b</td>
<td>64.7 ± 1.08^b</td>
<td>68.4 ± 1.35^b</td>
<td>3.10 ± 1.12^b</td>
</tr>
</tbody>
</table>

Numbers with different letters (a and b) in the same column are significantly different (P<0.05).
Table 2
Correlation between protein fractions of different molecular weight (Mr) and semen quality parameters.

<table>
<thead>
<tr>
<th>Band number</th>
<th>Mr (kDa)</th>
<th>pH</th>
<th>Motility (%)</th>
<th>Viability (%)</th>
<th>Concentration (10⁹ cell ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>116.20L</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>108.35H</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>78.58</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>72.45L</td>
<td>–</td>
<td>–</td>
<td>0.510*</td>
<td>0.377*</td>
</tr>
<tr>
<td>5</td>
<td>72.45L</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>6</td>
<td>49.78</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>7</td>
<td>44.89</td>
<td>–</td>
<td>–</td>
<td>0.462*</td>
<td>–</td>
</tr>
<tr>
<td>8</td>
<td>42.65</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>9</td>
<td>37.24</td>
<td>0.433*</td>
<td>–</td>
<td>0.392*</td>
<td>–</td>
</tr>
<tr>
<td>10</td>
<td>25.37H</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>11</td>
<td>23.19</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>12</td>
<td>21.48H</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>13</td>
<td>18.73H</td>
<td>–</td>
<td>–</td>
<td>0.469*</td>
<td>–</td>
</tr>
<tr>
<td>14</td>
<td>15.96H</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>15</td>
<td>15.13L</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

The letters L and H indicated the relative protein content (%) of this protein fraction in the low fertility group was significantly lower \( (P<0.05) \) and higher \( (P<0.05) \) than that in the high-fertility group, respectively.

* \( P<0.05 \).
** \( P<0.01 \).

L-group was considerably lower than that in the H-group. Protein band 4 was positively correlated with sperm viability and concentration, and protein band 15 was negatively correlated with sperm motility. The amount of five more protein bands (2, 10 and 12–14) in the L-group was significantly higher than that in the H-group. Three protein bands (2, 10 and 14) were negatively correlated with semen pH value. Protein band 10 was negatively correlated with sperm motility and protein band 14 with sperm viability and concentration. Protein band 12 was negatively correlated with sperm motility and concentration, while protein band 13 was positively correlated with sperm motility.

Although the relative protein content of another seven protein bands was not significantly different \( (P<0.05) \) between the H- and L-groups, they were also correlated with some semen quality parameters. Two protein bands (3 and 6) were negatively correlated with sperm motility. In addition, two protein bands (5 and 11) were negatively correlated with sperm concentration. Conversely, protein band 7 was positively correlated with sperm motility and protein band 8 with sperm concentration. Finally, protein band 9 was positively correlated with semen pH value and viability.

4. Discussion

In this study, one-dimensional SDS-PAGE was used to investigate the fertility-associated proteins and the correlation between specific seminal plasma protein and semen characteristics. Fifteen protein bands with molecular weights ranging from 15.13 to 116.20 kDa were detected in this study. The proteins with molecular weight below 26 were prominent (59% of the bands). In the study by Jobim et al. (2005), molecular weights ranged from 15 to 115 kDa and the most prominent spots were those <30 kDa. The protein spots with a molecular weight from 15 to 20 kDa accounted for 41% of the relative intensity of the spots of the gel. 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). Cardozo et al. (2006) 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. In the study by Bergeron et al. (2005), SDS–PAGE analysis of alcohol-precipitated ram seminal proteins indicated the presence of about 25 proteins with molecular masses from 14 to 120 kDa; a group of proteins with a molecular mass of 15–16 kDa and 22–24 kDa was more predominant. So we came to the conclusion that most proteins in ram seminal plasma are below 30 kDa. The difference of molecular weight could be influenced by season, breeds and collecting and preparation methods of seminal plasma.

Our results showed that the relative content of eight protein bands was different between the H- and L-groups. It is noteworthy that the 116 kDa protein band cannot be detected in most seminal protein
samples of the L-group and the relative content of this protein was much lower when compared to its content in the H-group. So the 116 kDa protein band could be a kind of protein or a group of proteins which was correlated with the ram fertility. The high relative protein content of band 14 (15.96 kDa) in the L-group could explain its negative correlation with sperm viability and concentration. This protein band could be the tissue inhibitor of metalloproteinase-2 (TIMP-2) mentioned by Jobim et al. (2005) and the 15.9 kDa protein observed by Cardozo et al. (2006), which was negatively correlated with sperm viability and more abundant in the non-breeding season. The relative content of band 4 (72.45 kDa) was more abundant in the H-group and positively correlated with sperm viability in the present study. Likewise, this protein in our research may correspond to the 73.2 kDa protein in the previous study (Cardozo et al., 2006). The content of this protein was reduced significantly in the non-breeding season and correlated with viability. However, the content of 72.45 kDa protein in our result was correlated with sperm concentration and this point was not observed by Cardozo et al. (2006).

In addition, the protein bands 2, 10 and 14 were negatively correlated with semen pH value and two of them (10 and 14) were negatively correlated with sperm motility, viability or concentration. We can conclude that the proteins that can dramatically decrease semen pH value are detrimental to the survival of spermatozoa. The previous studies have suggested that most proteins in seminal plasma of ram are acidic. These acidic proteins could contribute to the lower pH value (<7) of ram semen. In our result, the 37.24 kDa protein was positively correlated with pH value supporting the alkalinity of this protein.

Although the other seven protein bands of all seminal plasma samples showed no fertility-associated changes in their relative content, they were correlated with certain semen quality parameters. These findings suggest that some proteins may modulate sperm function by providing energy and protection for spermatozoa as a complementary substance. This point was also mentioned by Cardozo et al. (2006).

In conclusion, the present study shows that there is a significant difference in seminal plasma protein composition between low-fertility and high-fertility sheep. Most proteins are significantly correlated with semen characteristics. More importantly, this study indicates that the relative content of seminal plasma protein could be an essential index to evaluate ram fertility and semen quality. However, further studies and more experimental data are needed to construct reliable mathematical models which would make it possible to develop a simple method for the prediction of ram fertility.

Acknowledgements

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References