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Protein profile of serum and urine during early pregnancy in sahiwal cows

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ABSTRACT

Early pregnancy diagnosis is necessary to maintain reproductive efficiency in animals. To determine biomarker for the early pregnancy diagnosis, total protein was estimated and SDS PAGE was performed on urine and serum in selected 30 Sahiwal cows. Total serum protein concentration was increased in pregnant as compared to non-pregnant Sahiwal cows. A continuously increasing trend of protein was observed in pregnant group after 12 (6.36 ± 0.76 g/dl) to 22 (6.87± 0.39 g/dl) days post-breeding whereas non-specific trend was found in non-pregnant group. In urine, total protein concentration increased significantly in pregnant group as compared to non-pregnant group from 16 to 22 days. An increase in protein concentration from day 0 to 16 followed by decrease till day 22 was observed in both groups. Maximum and minimum protein concentration was observed on day 16 (31.6 ± 0.68 mg/dl) and day 0 (23.32 ± 0.81 mg/dl) respectively, in urine of pregnant group. Upon SDS PAGE analysis of serum, expression of specific proteins of molecular weight between 29 to 43 kDa with over expression of 66 kDa protein were observed in pregnant cows. It was observed that, proteins with molecular weight of 43 kDa and 66 kDa were over-expressed in pregnant cows as compared to non-pregnant cows. These findings suggest that pregnancy specific proteins ranging 29 to 43 kDa of pregnant Sahiwal cows can be used as pregnancy biomarker in near future.

Keywords: Biomarker, Bovine, Early pregnancy diagnosis, Sahiwal, SDS PAGE

Reproductive efficiency depends on the production of healthy calf within a year or 12 months period. It can be achieved by focussing on herd production and management issues like genetic selection, estrus synchronization, body condition score, specific fertility genomic markers, proper heat detection, male fertility management, semen evaluation, use of A.I., early and rapid pregnancy diagnosis and assisted reproductive technologies (Crowe *et al.* 2018). Early pregnancy diagnosis (cyesiognosis) is very important fertility management tool as it is the way to separate pregnant and non-pregnant animals at early stage. Many times, farmers keep rearing the non-pregnant animals which in turn affects their economics (Youngquist 1997, Duggal *et al.* 2001). Pregnancy diagnosis benefits farmers by timely culling of unproductive/ sterile animals, obtaining information about reproductive health of the animal and timely treatment of the infertility in the animals. It helps to maintain the pregnant cows and regulate calving for the production of calves throughout the year (Bekele *et al.* 2016).

Two methods widely used are, direct and indirect to identify pregnancy in bovine. Direct methods include transrectal palpation and transrectal ultrasonography. However, neither of these methods can identify pregnancy before the start of next estrus cycle (Cain and Christiansen 2015). Indirect methods include detection of early pregnancy factors, pregnancy associated glycoprotein, progesterone assay, interferon stimulated genes, bovine trophoblastic proteins, estrone sulphate concentration and circulating nucleic acid. Though methods like RIA, ELISA have been developed to detect progesterone, EPF and PAG but these methods are not 100% accurate.

Nowadays, proteomics has opened new avenues for exploring large number of pregnancy associated biomarkers. Proteomics has potential to analyze thousands of proteins in a single experiment from various body fluids (Lippolis and Reinhardt 2008). This will help in identifying pregnancy specific and sensitive biomarkers. Body fluids like blood, urine and milk have a wide range of abundant proteins and it has been observed that low abundance proteins have the maximum possibility of being the novel biomarkers (Colantonio and Chan 2005). Currently, limited information is available on the Sahiwal cow serum and urine proteins in relation to pregnancy. Therefore, present study was conducted to analyse and characterize proteins in serum and urine of Sahiwal cow during post breeding period to identify early pregnancy proteins.

MATERIALS AND METHODS

The research was conducted on healthy Sahiwal cows (30). The animals were kept in Instructional Dairy Farm, Pantnagar, Uttarakhand under uniform feeding and managemental conditions. After proper restraining of the cows, just before AI, 5 ml blood was collected in a clot

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activator tube from the jugular vein by using 18G needle and 10 ml urine was collected by either catheterization or massaging of the perineal area. Sampling was further done on 12, 16, 19 and 22 days of A.I.

Processing of the samples: Blood samples were centrifuged at 3000 rpm for 15 mins and serum was withdrawn. To the collected urine sample, double amount of acetone was added and samples was stored at -4°C for overnight. It was then centrifuged at 10,000 rpm for 20 min at 4°C. The precipitate was recovered and washed twice with chilled acetone followed by its drainage completely and air drying over ice. The precipitate left was dissolved in 1 ml PBS (Phosphate buffer saline). Serum and urine, both samples were stored at -20°C till further processing and analysis.

Total protein estimation by Bradford's method: Total protein concentration was estimated in both urine and serum samples by Bradford's method. It is done to determine the difference in total protein concentration of biological samples from pregnant and non-pregnant cows.

SDS PAGE: After protein extraction from both serum and urine sample, the protein was electrophorised by one dimensional SDS PAGE using 12% acrylamide gel which was run at 70V till the proteins leave the well and then at 100V for 4 hrs. After electrophoresis, gel was stained with Coomassie brilliant blue R250 for overnight, followed by destaining (2-3 times) with ethanol and acetic acid. The acrylamide gel was documented using Alpha imager documentation system.

Pregnancy confirmation: Pregnancy was confirmed by performing trans-rectal ultrrasonography on 30 days post breeding. Pregnancy was further also confirmed by trans-rectal palpation on 45 days post-breeding and accordingly animals were divided into two groups (Pregnant and non-pregnant cows).

RESULTS AND DISCUSSION

Transrectal ultrasonography: In transrectal ultrasonography, cervix was normal (hyperechoic) in both the groups. Uterine body was normal in both the groups while in the uterine horn of the pregnant animals amniotic vesicle (hypoechoic lining anechoic area) was visible along with fetal heartbeat, indicating viable embryo (hyperechoic area) (Fig. 1). All 30 animals were scanned, out of which 15 cows were found pregnant on the basis of USG. This indicates nearly 50% conception rate in Sahiwal cows.

Transrectal palpation: On transrectal palpation of the animals, cervix was found normal in size and tonic in both the groups. On further palpation, uterine body was found to be soft, pliable and relaxed with horns of the pregnant animals enlarged as compared to non-pregnant animals. Presence of the amniotic vesicle and slipping of the chorio-allantoic membrane was also observed in the pregnant animals and these findings were used to confirm the pregnancy. Out of all 30, 15 were found pregnant and indicated 50% conception rate.

Fig. 1. Pregnancy diagnosis by ultrasonography on day 30 after AI. Amniotic sac is visible in the ultrasound and the arrow indicates embryo inside amniotic sac.

Total protein: Total blood protein concentration in sahiwal cows on day 0, 12, 16, 19 and 22 post A.I. is presented in Table 1. The protein concentration was observed to be higher in pregnant animals on all days as compared to non-pregnant animals though difference was non- significant. However, an increase in the protein concentration of the pregnant cow was observed from day 0 to day 22 with maximum protein concentration on day 22 and minimum on day 0. In pregnant animals, the total protein concentration increased from day 12 to day 22 while the non-pregnant group showed no distinct pattern. The maximum concentration was observed on day 16 in the non-pregnant group and the minimum was observed on day 0.

Table 1. Total blood protein concentration in pregnant and non-pregnant Sahiwal cows (g/dl)

Days of estrous cycle	Pregnant (group I) (n=15)	Non-Pregnant (group II) (n=15)
0	5.84±0.65	5.28±0.69
12	6.36±0.76	5.66±0.55
16	6.39±0.64	5.84±0.48
19	6.41±0.65	5.78±0.41
22	6.87±0.39	5.47±0.49

Urine: The total protein concentration on day 0, 12, 16, 19 and 22 post A.I. is presented in Table 2. Total protein concentration was observed to be significantly higher on all

days as compared to non-pregnant animals with significant difference (P<0.1) between pregnant and non-pregnant cows on days 16, 19 and 22. An increase in the protein concentration of the pregnant cow was observed from day 12 to day 16 and then it decreased up to day 22. Maximum protein concentration in pregnant group was found on day 16 and minimum on day 0. In non-pregnant group, total protein concentration also showed same pattern. Significant difference (P<0.1) was observed between day 19 and 22 in the non-pregnant group.

Table 2. Total protein concentration (mg/dl) in urine of pregnant and non-pregnant Sahiwal cows

Days of estrous cycle	Pregnant (group I) (n= 15)	Non-Pregnant (group II) (n=15)
0	19.32 ± 0.81	18.22 ± 0.89
12	26.5 ± 0.59	21.88 ± 0.98
16	31.6 ± 0.68^{a}	24.43 ± 0.97
19	27.3 ± 0.79^{a}	22.79 ± 0.98^{ab}
22	25.9 ± 0.74^{a}	20.48 ± 0.68^{ab}

Means bearing different superscripts 'a' in a row differ significantly (^{a}P<0.1) indicating difference between groups and 'b' in a column differ significantly (^{b}P<0.1) indicating difference between days within groups.

Protein characterization by One-Dimensional SDS PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

Protein characterization was done by SDS PAGE and results obtained are presented in Figs. 2-4. Urine and serum were analyzed in SDS PAGE after protein extraction. Approximately 35 µg protein was loaded and electrophoresed and size fractionated against the protein ladder of molecular weight from 14.3 kDa to 97.4 kDa.

Blood: Upon analysis of SDS PAGE of blood protein, different bands were observed ranging in molecular weight from 14.3 kDa and 97.4 kDa. Darker bands were observed between 20.1 kDa and 29.0 kDa while light bands of molecular weight between 14.3kDa-20.1 kDa, 29.0 kDa - 43.0 kDa and 43.0 kDa-66.0 kDa were observed at all days in both pregnant and non-pregnant animals. As per the Fig. 2, different bands of molecular weight ranging in between 35.0 kDa and 43.0 kDa were observed from day 16 to day 22 in pregnant cows. In the non-pregnant animals these bands were absent. The protein band of molecular weight 66.0 kDa was consistently observed at all days in both groups.

As observed in Fig. 3, different bands of molecular weight between 35.0 kDa and 43.0 kDa was observed on day 22 only, which might be due to individual variations. Overexpressed band of molecular weight 66.0 kDa was also observed on day 19 and 22 in the pregnant cow. Specific changes in the protein bands of the non-pregnant animal were not observed.

Urine: Earlier it was believed that proteins are not present in the urine. But later it was found that it is present in the urine in little amount and increases only during any specific physiological or pathological conditions. Before

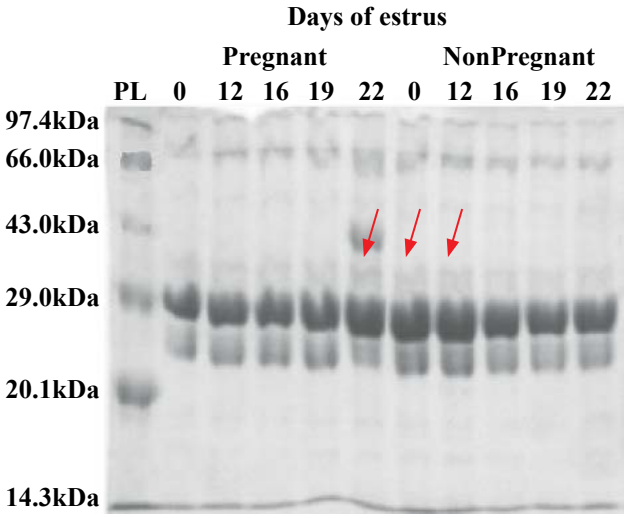

Fig. 2. SDS PAGE analysis of the serum sample isolated from pregnant and non-pregnant cows.

performing SDS PAGE, urine was precipitated by acetone. Sudha *et al.* (2012) reported yield of buffalo urinary protein by acetone precipitation at 50% saturation was 1.1% while it was 0.156% by ammonium sulphate precipitation at 90% saturation. In buffalo urinary proteins, it was reported that acetone yielded higher amount of protein precipitation in comparison to TCA-Acetone, ammonium sulphate and sodium chloride precipitation (Sharma 2014).

After performing SDS PAGE, different protein bands were observed between molecular weight 14.3 kDa and 97.4 kDa. Dark bands of molecular weight between 29.0 kDa and 43.0 were observed and light bands between 43.0 kDa-66.0 kDa and 20.1 kDa-29.0 kDa were observed in both pregnant and non-pregnant cows. In Fig.4., band of molecular weight 43.0 kDa was observed from day 12 onwards and the maximum intensity was seen on day 19. Furthermore, the expression of the band of molecular weight 66.0 kDa was more from day 16 onwards in the

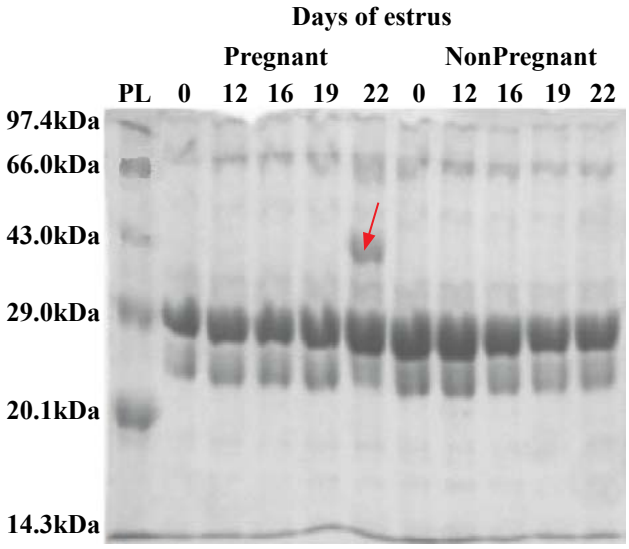

Fig. 3. SDS PAGE of the serum sample isolated from pregnant and non-pregnant cows.

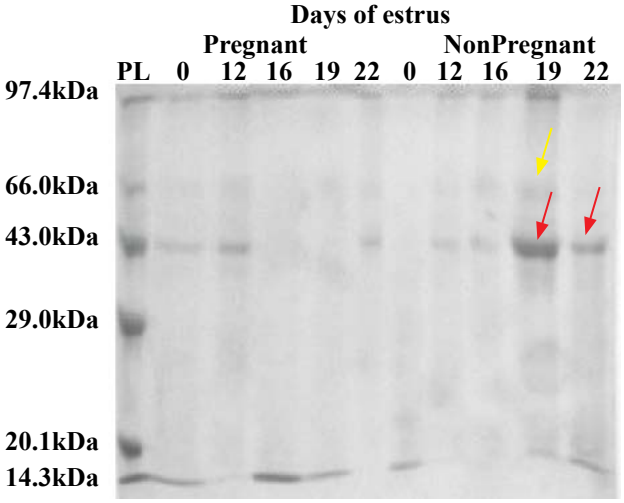

Fig. 4. SDS PAGE of the urine sample isolated from non-pregnant and pregnant cows.

pregnant animals. In all days of both the groups, band of molecular weight 43.0 kDa was over-expressed as compared to other bands. No specific bands were observed in the pregnant cow as compared to non-pregnant animal.

Urine is formed through glomerular filtration of the plasma in the kidney. Plasma filters and some of the proteins are excreted in the urine hence, act as remains of the proteins of the plasma (Bathla *et al.* 2015). As the concentration of the proteins is very less in urine therefore, the bands obtained in SDS PAGE are faint as compared to the bands in the serum.

Findings of the ultrasonography are in accordance with Filteau and DesCoteaux (1998) who stated that pregnancy diagnosis by ultrasonography can be done as early as 25-28 days after breeding. Under controlled environment and using a high-quality scanner and transducer fetal heartbeat can be visualized at around day 21 of gestation, confirming viable pregnancy. Sensitivity and specificity of pregnancy diagnosis using ultrasound was 44.8% and 82.3%, respectively, when conducted between day 21 and 25 of breeding whereas it increased to 97.7% and 87.7%, respectively, when conducted between day 26 and 33 of breeding (Pieterse *et al.* 1990). Ultrasonography also provides best evaluation of fetal viability, fetal age and the most accurate assessment of twin pregnancies starting from day 25 of breeding (Fricke 2002).

Per-rectal palpation of amniotic vesicle (spherical, turgid, fluid filled structure) is an aid in determining pregnancy status in cattle. After day 30 of gestation, slipping of the chorioallantoic membranes between thumb and forefinger, asymmetry of the uterine horn with thinning of its wall may be used for diagnosis of pregnancy (Zemjanis 1970). But accuracy at 30 days post breeding is less, approximately 54% therefore transrectal palpation in present study is done on day 45, which has accuracy of around 95-99% (Gunn and Hall 2018) and 93.8% (Jaskowski *et al.* 2019).

The findings in our results are in accordance with Abdullah *et al.* (2017), as he also observed non-significant difference in plasma protein concentration between pregnant and non-pregnant in cross-bred and zebu cows. The maximum concentration however, observed on day 30 of gestation was 6.59 and 6.95g/dl in crossbred and zebu cows respectively. It was observed that total plasma protein amount was higher in mid and late gestation as compared to non-pregnant cross bred cows (Mir *et al.* 2008). Similarly, increasing pattern with total protein during pregnancy was observed in human maternal serum starting from day 12 of pregnancy (Mc Donald 1980, Lathura *et al.* 1987). It was observed by Bhoraniya *et al.* (2012) that the concentration of total plasma protein was 5.93g/dl in pregnant cows but non- significant difference between pregnant and non-pregnant Kankrej cows.

As compared to normal concentration of protein in urine, the concentration obtained in our study was higher. It has been reported that urine after precipitation with acetone yields higher amount of protein concentration as compared to the untreated urine (Bathla *et al.* 2015). However, trend (increasing from day 12-16 and then decrease by day 22) obtained in this study was similar to the findings of the other authors (Manzoor 2013, Salam 2016). Salam (2016) reported total urinary protein in pregnant cross bred cows to be 3.49±0.11 to 12.21±0.23 mg/dl which was significantly higher ($P<0.01$) than non-pregnant cows (2.69±0.26 to 3.05±0.37 mg/dl) on days 12, 14 and 16. It is observed that many metabolic changes occur during early pregnancy that has effect on kidney filtration, leading to more protein concentrations in the urine. This may be due to physiological changes like increasing glomerular size and GFR (Ibeh 2006). Katiyar (2012) reported the concentration of the total protein in pregnant buffalo varied from 5.37±0.31 to 15.83±0.23 mg/dl, which was significantly higher ($P<0.01$) than non-pregnant buffalo (5.09±0.18to 11.61±0.37 mg/dl). Manzoor (2013) reported that total protein concentration in urine of pregnant buffalo was higher than non-pregnant buffalo i.e. 6.22±0.39 to 12.56±0.20 mg/dl and 2.24±0.05 to 3.47±0.44 mg/dl, respectively. Abdullah *et al.* (2017) also observed significantly higher ($P<0.05$) urinary protein concentration in pregnant zebu cows as compared to non-pregnant and an increase in protein concentration from day 15 to 24.

On SDS PAGE of serum, PAG of 67.34 kDa molecular weight was specific in pregnant animals (Lestari and Ismudiono 2011). PAG was observed in serum of the pregnant cow with molecular weight of 67 kDa (Xie *et al.* 1994, Green *et al.* 2000). PAG could also be detected in maternal blood circulation at the time of embryo implantation and their molecular weight varied from 54-70 kDa (Kiewisz *et al.* 2008). In a study, SDS PAGE was done on the placenta of moose and elk. In placenta of moose, PSPB (mPSPB) of molecular weight 58 kDa and 31 kDa while in elk, PSPB (ePSPB) of 57 kDa, 45 kDa and 31 kDa were observed. It has been reported that moose and elk have properties similar to that of bovine and ovine PSPB hence, it can be used for further studies (Huang *et al.* 1999). SDS PAGE of placental fluid of the six synepitheliochorial

species (cattle, elk, bison, buffalo, sheep and goat) revealed the highly stained proteins of 45 and 66 kDa molecular weight (Bella *et al.* 2007). In other study, protein band of molecular weight of 55 kDa was observed in the blood of goats which was identified as PAG (Ningtyas *et al*. 2019). Proteins of molecular weight 42 kDa and 52 kDa were over-expressed on day 21 and 28 days post A.I. in pregnant goats (Inyawilert *et al.* 2019). In an experiment on serum of pregnant buffalo, two pregnancy specific proteins of 70 kDa and 30 kDa molecular weight were reported (Balamurugan *et al*. 2020).

Salam (2016) observed over-expression of 66 kDa protein band in pregnant buffalo as compared to non-pregnant buffalo. In a study on the urine of pregnant HF cows, proteins bands of molecular weight 20 kDa and 24 kDa were observed in the pregnant animals and 27 kDa and 28 kDa in non-pregnant animals (Hwang and Lim 1999). Pyo *et al.* (2003) isolated 21 kDa protein with 6.1 p.I. from cow urine and named it bovine pregnancy associated protein (bPAP). It was reported that expression of 66 kDa protein band was more in pregnant buffalo as compared to non-pregnant buffalo (Sharma 2014). Up-regulation of 5 protein bands were reported in urine of pregnant buffalo as compared to non-pregnant (Jithil 2015).

Total proteins concentration was influenced by the pregnancy in cows. It increased in the pregnant Sahiwal cows compared to non-pregnant cows. Further, protein bands observed in both urine and serums have similarities from day 16 to 22 post-breeding. Protein bands of 29.0 kDa and 43 kDa might be specifically associated with pregnancy and can further be analyzed to use as early pregnancy marker in Sahiwal cows.

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