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Purification and Bioactivity of Placental Lactogen from Water Buffalo, *Bubalus bubalis*

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ABSTRACT

Mammalian placental lactogen, also known as chorionic somatomammotropin is crucial in controlling the growth of mammary tissue during pregnancy and its release during pregnancy accounts for increased mammogenesis. Bubaline placenta was obtained and processed for extraction of extracellular proteins at pH 9.5 using Tris-Glycine buffer. Placental lactogen was purified by gel filtration chromatography using Sephadex-G100 and ion exchange chromatography on DEAE- Sepharose, employing a linear salt gradient. SDS-PAGE was used to monitor the purification steps, and protein band was located by immunoblotting technique. Bubaline placental lactogen was purified to homogeneity and showed a molecular weight of about 30 kD. Purified placental lactogen was administered to male albino mice for up to eight weeks for growth promoting activity. Increased mammary growth was observed in test female non lactating mice, as compared to controls when placental lactogen was applied. Increase weight gain for visceral tissues like kidney, liver, muscle and lungs was also observed, as compared to control subjects. It was also recorded that the exogenous supply of bubaline PL reduces fat cells in the hypodermal layer of skin.

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Authors' Contribution
AJS designed the experimental work and analyzed the results. RS and SM helped in experimentation. ARS and MK helped in preparation of manuscript.

Key words
Placental lactogen, *Bubalus bubalis*, Water buffalo.

INTRODUCTION

Placental lactogen (chorionic somatomammotropin), is a placental polypeptide hormone. The structure and function of placental lactogen is closer to that of growth hormone (somatotropin). It facilitates the energy supply of fetus by regulating the metabolic state of the mother during gestation. Placental lactogen belongs to prolactin-growth hormone family Placental lactogen was purified and characterized first time by Buttle *et al*. (1972) in grazing mammals namely goats, sheep, and cows, *etc.* Ovine placental lactogen has been identified and purified and its characterization indicates a 23kDa protein with 198 amino acids and three disulfide bonds. The molecule is non-glycosylated naturally consisting of a single chained structure with low molecular weight (Byatt *et al*., 1992).

Placental lactogen is secreted throughout pregnancy, but the levels vary in both maternal and fetal circulation and among species, *e.g.,* in dairy animals the levels are low as compared to those present in ewes (Byatt *et al*., 1987). Placental hormones and fetal–placental development was reviewed by Gootwine (2004). Leibovich *et al*. (2000) studied the functional role of oPL in pregnancy and lactation in ewe lambs. Gertler and Djiane (2002) reported the mechanism of ruminant placental lactogen action: molecular and in vivo studies. Ovine placental lactogen-induced heterodimerization of ovine growth hormone and prolactin receptors in living cells (Biener *et al*., 2003). Crystal structure and site 1 binding energetics of human placental lactogen has been reported by Walsh and Kossiakoff (2006). The anticipated cDNA shares sequence homology with various other milk producing animals. Placental lactogen has its biotechnological importance in farm industry, as its exogenous supply can improve the growth rate and productivity among ruminants (Leibovich *et al*., 2001). There are reports on the placental lactogen from several farm animals like cow, sheep, pig and goat (Bolander and Fellows, 1976; Bečka *et al*., 1977), there is no report on placental lactogen from water buffalo. Earlier we had reported the growth promoting the activity of bubaline placental protein extract (Sami *et al*., 2014). Here we report the purification and properties of bubaline placental lactogen.

MATERIALS AND METHODS

Isolation and purification of bubaline placental lactogen

Bubaline placenta was obtained from a local butcher shop from suburbs of Lahore. The tissues were, immediately washed with distilled water and stored at -20°C in a freezer,

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in small portions until further use. For experimental studies, 25 g of the placental sample were ground in 200 ml Tris-Glycine buffer (pH 9.5) in a food mincer. The suspension was passed through a thick sieve to remove cell debris, and the extract was employed for further studies. The crude protein extract was centrifuged at 10,000 g for 10 min, and the supernatant was obtained. For the removal of proteinous matter, the extract was precipitated with 80% acetone at 0°C and incubated at 20°C, for 24 h. The precipitated proteins were isolated by centrifugation at 10,000 rpm for 10 min. The pellet was dissolved in 20 ml buffer pH-9.5. Proteins were fractionated on a gel filtration column using SephadexG-100 column (1.8x30 cm). An-Ion exchange chromatography on DEAE-Sepharose was performed at pH 9.5 using a salt gradient ranging 0.01 to 0.5 M (column size 0.8x15 cm). The proteins were eluted at the flow rate of 0.5 ml/min. A total of 100 fractions (1 ml each) were collected, Protein estimation was done by reading absorbance at 280 nm ad Bradford assay. Eluted fractions were analyzed for immunogenic activity against ovine placental lactogen antibody (Abcam USA) and SDS-PAGE (Sami and Shakoori, 2014).

Bioassay for bubaline placental lactogen

After the purification of BbPL by chromatography, albino mice were used as a model organism to perform Bioassays. Bioassays were performed as described by Sami *et al*. (2014), for seven weeks. To monitor the effect of exogenous bubaline placental lactogen, on visceral tissues, mice were killed, and organs (lungs, liver and muscle), were removed. The organs were weighed and compared with the control. The experiment was carried out in triplicates and mean error of +3 was allowed. To check the effect of exogenous placental lactogen, on the female mice, growth of mammary glands and impact on mice dermal tissues was monitored. Three sets of female mice were injected with bubaline placental lactogen as described above. After ten days animals were killed and mammary glands were removed, after dissection. The tissues were stored in formalin till further studies. Formalin preserved Tissue samples were stained with Hematoxylin and eosin (H&E) staining technique (Thompson and Hunt, 1966; Sheehan and Hrapchak, 1980). Growth and proliferation of mammary glands and skin, in control and subjects, were analyzed by histological techniques.

RESULTS AND DISCUSSION

Placenta sample was collected from a butcher shop, the weight of placenta was about 4.50 kg. The tissue sample was stored at -20ºC. Twenty five g of placental tissue was grounded in pH 9.5 buffer and filtered out. Protein was isolated and estimated to be 3.2 g protein/100g tissue sample.

Proteins were concentrated using chilled acetone, with 80 % recovery. The proteins were fractionated on Sephadex G 100 column (1.8x30 cm). 50mg of protein was loaded onto the column, and 80 fractions were collected (1 ml each) at a flow rate of 0.5 ml per min proteins were estimated by Bradford method and U.V absorbance at 280 nm (Fig. 1). Fractions showing maximum protein concentrations were subjected, to SDS-PAGE (Fig. 3). 15 mg of protein solution was loaded in the ion exchange chromatography column packed with DEAE Sepharose using a linear salt gradient. A single broad peak was observed at 0.02-0.025 M NaCl gradient (Fig. 2). The peak fractions were also subjected to dot blot analysis for the confirmation of immunogenic activity (Fig. 2).

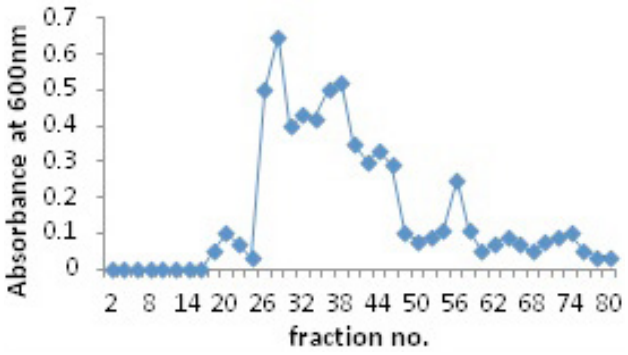

Fig. 1. Gel filtration of crude placental extract. 50mg of protein was loaded on Sephadex G 100 column (1.8x30 cm). 80 fractions were collected (1ml each) at a flow rate of 0.5ml per min.

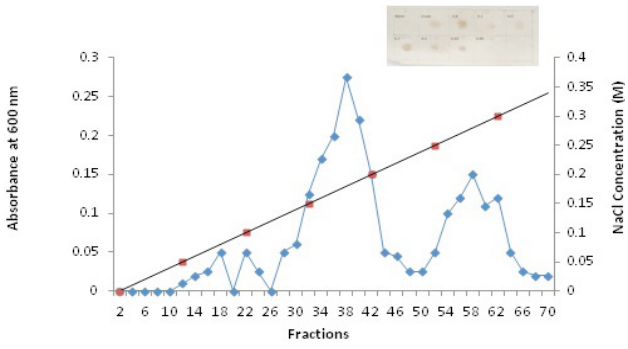

Fig. 2. Ion exchange chromatography of placental proteins of *Bubalus bubalis* isolated at pH 9.5. 15 mg of protein was loaded onto a column packed with DEAE-Sepharose (0.8x15cm); using a salt gradient 0.01-0.5M NaCl in seat shows dot blot analysis of peak fraction indicating presence of bPL. Fractions from peak No. 1 showed immunogenic activity against placental lactogen antibody (Abcam, USA) (Fraction No. 28-48).

After the confirmation and presence of bPL in selected portions, the SDS-PAGE gel was run. Single band at ~30 kDa position was observed identical to the position of a band on the immunoblot (Fig 3, Lane 1). Placental lactogens (PL) have been isolated and purified from several organisms including mouse (Colosi *et al*., 1982) sheep and cow (Martal and Dijane 1975; Hurley *et al*., 1977).

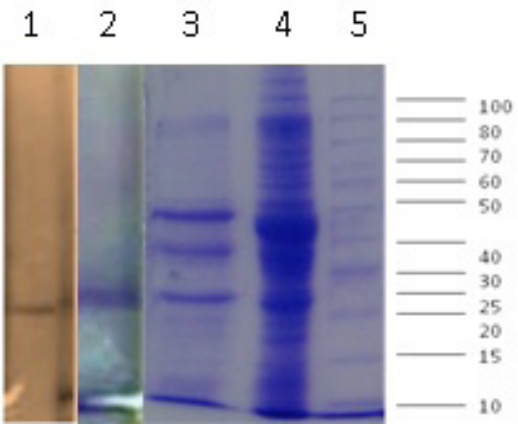

Fig. 3. 10% SDS-PAGE and Western blot anlaysis of placental lactogen. Lane 1, western blot; Lane 2, purified bPL; Lane 3, partially purified bPL (gel filtration); Lane 4, crude extract; Lane 5, protein marker.

Bovine PL was first isolated and purified from the placental tissue by Bolander and Fellows (1976). The molecular weight of the purified protein was around 30 kDa, possibly due to high glycosylation (Placental proteins are heavily glycosylated, perhaps to stabilize the structure of the newly synthesized proteins (Sami et al., 2014)). Results are shown in Figure 3, with an estimated molecular weight of 30 kDa. Murthy et al. (1982) detected one form of bPL at 30,000-32,000 Da MW and 5.5 PI. Several laboratories have prepared recombinant bPL, oPL (Sakal et al., 1997), and cPL (Sakal et al., 1998) in sufficient amounts. The native molecule contains both O-linked and N-linked oligosaccharides in its structure and this make it a higher MW molecule of 31,000-33,000 Da (Shimomura and Bremel, 1988). There is no indication that Pls of sheep, goat and human are glycosylated. This explains why the molecular weight of these PLs are lower (approximately 22,000) compared to that of bPL (Chan et al., 1976; Alvarez-Oxiley et al., 2008). Molecular weight of bubaline PL is comparable to Bovine PL possibly due to high glycosylation.

There are reports that the enzymatic removal of N-linked sugar from native bPL increased the binding affinity of somatotropin receptor for BPL by about 1.2-2.3 fold. However, removal of o-linked oligosaccharide had a small effect on either somatogenic or lactogenic binding (Byatt *et al*., 1992).

Growth promoting effect of purified bubaline PL was assessed using mouse models. Six controls and six subject mice were taken for bioassays. The animals were monitored to identify any signs of antigenicity or disease. Total body Weight and development were also checked. Purified Bubaline placental lactogen was tested for somatogenic activity in mice. The test animals were about 1.3 times bigger than control subjects when treatment was carried out for seven weeks (Fig. 4). Earlier we have reported that the placental peptide of ruminants has growth promoting activity in mammals (Sami et al., 2014; Gootwine, 2004). After three weeks the animals were dissected, and various body organs such as liver, kidneys, lungs were weighed and compared with untreated mice. It was indicated that on the average the liver of subjects was approximately 30% larger than the control subjects (Fig. 5). Similarly, the weight of kidney and muscles also increased for injected animals, 30% as compared to the control subjects. For lungs, there was an increase of around 27% for the subject in contrast to the controls (Fig. 6).

Another set of female mice were injected with purified BbPL and after ten days dissected, and mammary glands were examined for development. Furthermore, immunohistological staining was used for histological analysis of mammary glands.

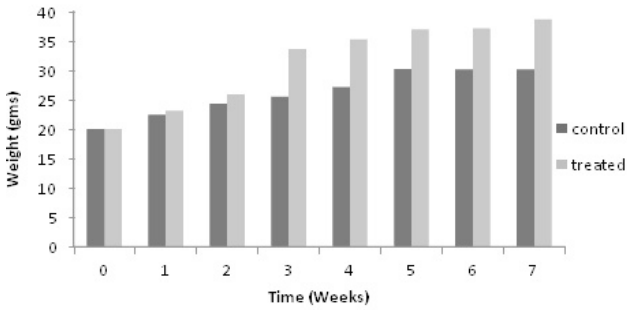

Fig. 4. The effect of exogenous placental lactogen on mice for seven weeks. The bars indicate increasing trend of weight in subjects over controls starting from 2nd week. The subject animals were injected with the hormone 3 µg daily. The controls were injected with the buffer without placental lactogen. The experiment was carried out in triplicates, mean error of ±3 was allowed.

The size of lobule and the lactiferous duct also increased in the test animals as compared to control (Fig. 7). Recombinant bovine placental lactogen has been shown to stimulate total mammary DNA in dairy heifers, but this effect does not occur in groups treated with a high level of rbPRL (Byatt and Robert, 1995). It was also observed that both hormones induce mammary differentiation, although bPRL appears more potent than

BPL (Hurley et al., 1980). Further evidence indicated that PRL is required for lactogenesis rather than for mammary growth in periparturient cows (Leibovich et al., 2001; Collier et al., 1993).

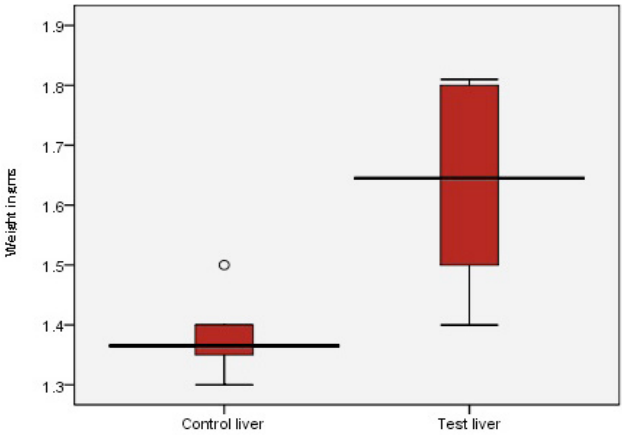

Fig. 5. Effect of purified bubaline placental lactogen on liver in mice. Noticeable size and weight increase was observed in liver.

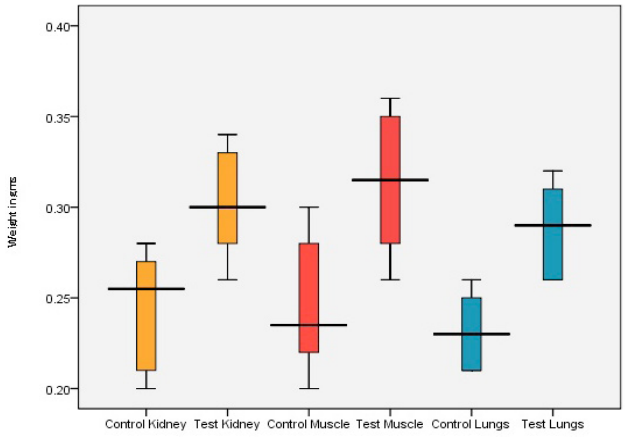

Fig. 6. Effect of purified bubaline placental lactogens on vital organs. Significant weight increase is observable.

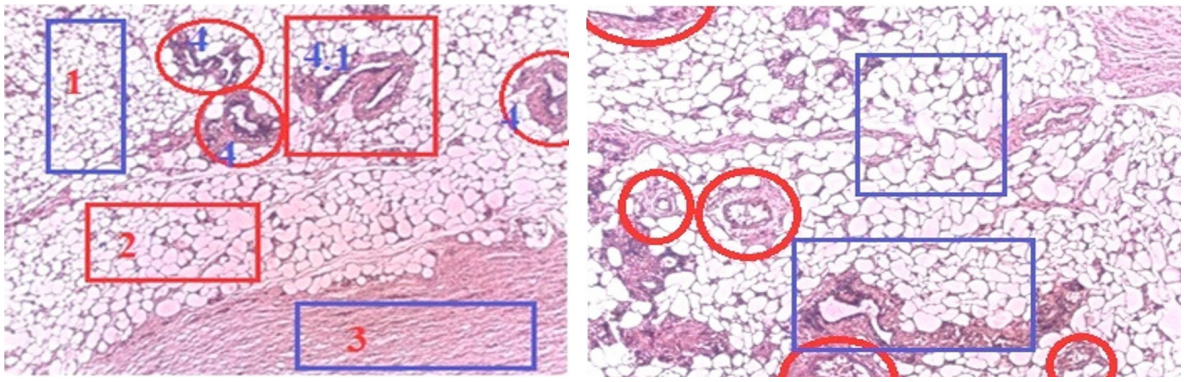

Fig. 7. Cross section of Mammary gland tissue of non-lactating female control mouse (left) and test (right). Magnification is 1000x power of light microscope. 1 and 2 indicates an increase in adipose tissue with increased number of cells as compared to the control. Three circles in red color labeled as 4 are lobule (alveoli) of mammary glands, while the red color square marked as 4.1 is lactiferous duct.

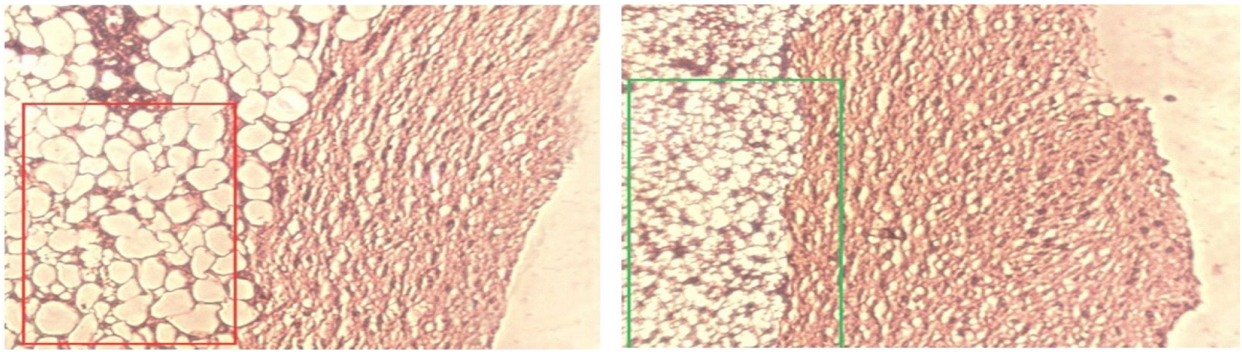

Fig. 8. Cross section of skin tissue of non-lactating female control mouse (left) and test (right). H & E stain: 1000x magnification. Red square indicate the larger and more adipocytes in hypodermal layer.Green square indicate the smaller size of adipocutes in hypodermal layer, under the influence of exogenous bPL with lesser fat content.

Examination of the skin of the control and test animals showed that there were more adipocytes with an increase in size in the hypodermal layer in the control subjects (Fig. 8, square indicated at left side). For test animals (Fig. 8, square indicated on right side) the smaller size adipocytes in the hypodermal layer, under the influence of exogenous BPL, indicating less fat contents.

These results suggested that BPL stimulates mammogenesis (Fig. 7). Results confirm the earlier findings given by Ferreira *et al*. (2013). This data support the hypothesis that BPL is one of the factors that is involved in mammary gland development.

CONCLUSION

Bubaline placental lactogen is glycosylated similar to bovine placental lactogen, as it has molecular weight closer to bPL. Exogenous supply of bubaline placental lactogen has growth promoting activity and has a biotechnological importance for improvement in milk and meat production.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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