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Glycoproteins profiles of placental extract -A step towards development of biomarkers of pregnancy in water buffalo

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Placenta synthesizes number of glycoproteins during pregnancy which come into the maternal circulation and can serve as biomarkers of pregnancy. Binucleate trophoblast giant cells (BNC) facilitate the transport of the glycoproteins across synepitheliochorial placental barrier (Wooding and Flint 1994). The BNC secreted pregnancy associated glycoproteins (PAGs) constitutes a large family of glycoproteins specifically expressed in the outer epithelial cell layer (chorion/ trophectoderm) of the placenta in several eutherian species (Butler *et al.* 1982, Green *et al.* 2000). In bovine BNC granules, PAGs are the main glycoproteins to which lectins bind (Klisch and Leiser 2003, Klisch*et al.* 2005). This feature of lectin is used to isolate PAGs selectively from placental extract by affinity chromatography in different species (Sousa *et al.* 2002, Klisch *et al.* 2005, Kiewisz*et al.* 2008, 2009, Barbato*et al.* 2008). Lectin affinity chromatography, therefore, may be useful for the isolation of such glycoproteins. Therefore, in the present study, attempts were made to isolate glycoproteins from cotyledons during different stages of pregnancy in buffalo using wheat germ agglutinin (WGA) lectin affinity chromatography.

Uteri (12) of pregnant buffaloes were collected from local abattoir. Age after removal of conceptus was estimated by measuring the crown-rump length (Richardson 1980). Based on the age of the conceptuses, pregnant uteri were divided into group 1 (up to 50 days), group 2 (51–100 days) and group 3 (>100 days).The fetal cotyledons of all the 3 groups were minced and homogenized separately. The extraction of protein was done as per Zoli *et al.* (1991). The pellet obtained after the centrifugation was discarded and supernatant of cotyledons was termed as placental extract (PE). After ammonium sulphate precipitation (fraction between 40 and 80% saturation) the proteins were dialyzed against Tris-buffer (0.01 M Tris- HCl buffer, pH 7.6) and loaded onto a WGA lectin affinity chromatography column. Unbound proteins were washed away with binding/ wash buffer. The bound glycoproteins were eluted by adding elution buffer and concentrated.The isolated glycoproteins from all the 3 groups were analyzed by 1 dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) as per Laemmli (1970).The hyper immune sera against the isolated glycoproteins were raised in rabbits as per Hudson and Hay (1989). Double immunodiffusion (DID) test was carried out to know the specificity of the hyper immune sera against placental extract and the non-pregnant tissue extract (buffalo muscles, kidney, liver and plasma) as per Ouchterlony and Nilsson (1978). Western blot analysis was done (Towbin*et al.* 1979) to know the immunoreactivity of the separated glycoproteins by SDS PAGE.

The concentration of protein extracted from different stages of pregnancy in the group 1, 2 and 3 were 0.3, 1.6 and 2.75 mg/g of cotyledonary tissue, respectively. The concentration of protein increased with the advancement of the stages of pregnancy which corroborated with the findings of other workers (Zoli*et al.* 1991, Sousa *et al.* 2002, Singh *et al.* 2005, Barbato *et al.* 2008). Similarly, Barbato *et al.* (2008) extracted 1.4 and 1.6 mg proteins/g from cotyledonary tissue of mid- and late- pregnancy in water buffalo. A different approach using lectin WGA affinity chromatography was used to isolate glycoproteins from PE during different stages of pregnancy in buffalo. The hypothesis behind using lectin affinity chromatography was that the binding of WGA is mainly restricted to the binucleate cells (BNC) granules (Klisch *et al.* 2010). It is likely that pregnancy-associated glycoproteins produced by BNC are enriched by this affinity chromatography (Klisch and Leiser, 2003). Klisch *et al.* (2010) used 24 different lectins for histochemical staining of placenta in 9 species and revealed affinity of WGA to BNC.In the present study, to the best of our knowledge, we have used WGA lectin affinity chromatography for the first time to isolate glycoproteins from placenta. Many workers have also used different lectins affinity chromatography to purify the PAGs from placentas of different species. Sousa *et al.* (2002) successfully identified PAG molecules after pepstatin affinity chromatography of cotyledonary extracts from the

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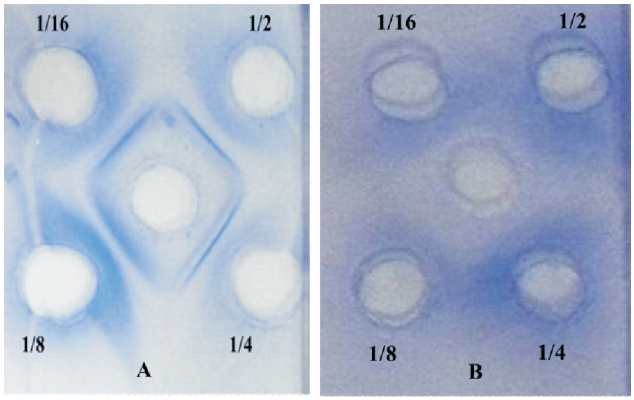

Fig. 1. Double immunodiffusion (DID) tests: placental (A) and non-pregnant tissue extract (B) were placed in central well separately.The peripheral wells were loaded with anti-glycoproteins at the specified dilution. The continuity of the precipitation lines suggests immunological identity.

zebu cattle (*Bos indicus*) placenta. PAGs were also isolated using a protocol which included *Vicia villosa* agglutinin (VVA) affinity chromatography from placenta of cattle (Klisch *et al.* 2005), American bison (Kiewisz *et al.* 2008), buffalo (Barbato *et al.* 2008) and European bison (Kiewisz *et al.* 2009).

The hyperimmune sera raised against the isolated glycoproteins reacted clearly with placental extract in DID test, but as such no cross reactivity could be observed with non-pregnant tissue extract (Fig.1) indicating presence of pregnancy associated glycoproteins in the isolated glycoproteins by WGA affinity chromatography method. El Amiri *et al.* (2003) also used DID as a tool to identify the PAGs and found antisera raised against PAGs clearly react with placental extract and no placental extract reacted with anti-BSA. The hyperimmune sera were used further to identify immunoreactivity of the various glycoproteins separated by SDS-PAGE by western blot during different stages of gestation in buffalo.

SDS- PAGE was carried out to separate the glycoproteins isolated through WGA lectin affinity chromatography during different stages of gestation. The electrophoretic profiles and immunoblot of proteins at different stage of gestation are presented in Fig. 2. The results revealed presence of 4 glycoproteins of 86, 67, 56 and 51 kDa molecular weights in group 1. Out of 4 glycoproteins; 3 glycoproteins (67, 56 and 51 kDa) were immunoreactive on western blot analysis. Five glycoproteins of 86, 75, 67, 56 and 38 kDa molecular weights were identified in group 2 and 3 of PE and all were found immunoreactive on western blot analysis which is in agreement with the finding of others (Singh *et al.* 2004, 2005, Klisch *et al.* 2005, Barbato *et al.* 2008). Klisch *et al.* (2005) also reported 3 most common PAGs bands of approximate 75, 66 and 56 kDa. Green *et al.* (2005) reported bovine PAGs purified from placenta which showed a nearly similar band pattern (75, 65 and 55 kDa). In the present study, there were no differences in glycoprotein profile purified from groups 2 and 3 of PE which is in agreement to the finding of Klisch *et al.* (2005).

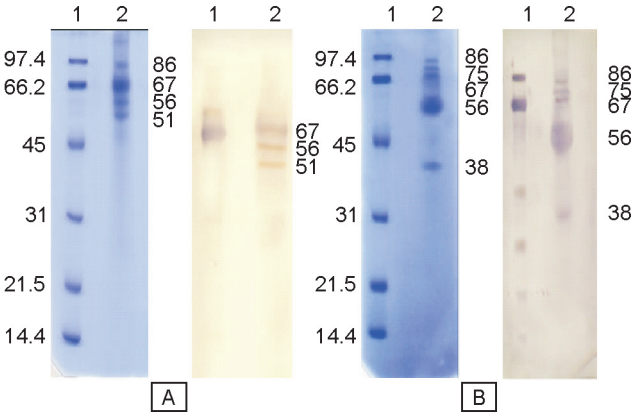

Fig. 2. Representative polyacrylamide gel electrophoresis and western blot of cotyledonary glycoproteins precipitated at 40–80% ammonium sulphate saturation. Each experiment was repeated 3 times. Lane 1 and 2 are molecular weight marker (kDa) and glycoproteins respectively.**A.** Electrophoretic profile and immunoreactive glycoproteins isolated from placental extract (up to 50 days pregnancy) of buffalo. **B**. Electrophoretic profile and immunoreactive glycoproteins isolated from placental extract (> 50 days pregnancy) of buffalo.

This finding is also consistent with the temporal expression pattern of PAG mRNAs which showed major changes of PAG expression occur only in early pregnancy and near term (Green *et al.* 2000).

SUMMARY

In this study, profiles of glycoproteins of buffalo cotyledons at different stages of pregnancy were studied. First time, WGA lectin affinity chromatography was used to isolate glycoproteins from cotyledons at different stages of gestation in buffalo. The hyper immune sera against the isolated glycoproteins showed no cross reactivity to non-pregnant tissue extract confirming the presence of glycoproteins in samples associated with pregnancy. During early pregnancy, presence of 4 glycoproteins of molecular weights 86, 67, 56 and 51 kDa and out of these glycoproteins, 3 glycoproteins (67, 56 and 51 kDa) were found immunoreactive on western blot analysis. Five glycoproteins of molecular weights 86, 75, 67, 56 and 38 kDa were identified in the mid- and late- stages of pregnancy and all were found immunoreactive. These glycoproteins may be useful for development of RIA/ ELISA based species specific assay for pregnancy diagnosis and monitoring of fetus in buffalo.

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