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Pregnancy-associated glycoproteins (PAGs) concentrations in water buffaloes (*Bubalus bubalis*) during gestation and the postpartum period

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ABSTRACT

For the first time in literature this study describes the pregnancy-associated glycoprotein (PAG) profile of buffalo cows during gestation and the post-partum period using antiserum raised against PAG-molecules purified from buffalo placenta (AS#860).

Ninety-eight buffalo cows, belonging to a buffalo herd subjected to a synchronization and artificial insemination (AI) program, were enrolled in this study. Blood samples were taken on days 0 (AI), 23, 25, 28, 30 and then biweekly until the end of pregnancy. Pregnancy was confirmed by ultrasonography on days 28 and 45, and by rectal palpation from day 60 onwards. Blood samples were suspended for the non-pregnant cows on day 45, while the blood of 20 buffaloes that had calved was tested every five days from the day of calving until day 50 post-calving. A cut-off value of 1.0 ng/mL was used in order to discriminate between pregnant and non-pregnant buffaloes. We used Linear Mixed models after Log(x+1) transformation to analyse the PAG concentrations.

Fifty-two buffalo cows had become pregnant out of 98 synchronized (53%) and 46 remained non-pregnant (47%) as shown by ultrasonography and the PAG analysis. Significant differences ($P < 0.001$) in PAG concentrations were observed between the pregnant and non-pregnant buffaloes from day 23 as the PAG of the non-pregnant cows was always close to zero. Conversely, the PAG of the pregnant cows increased progressively from day AI until day 105 post-insemination and then stabilized until the end of pregnancy. Regarding pregnancy diagnosis, the sensitivity of PAG-RIA 860 system (ability of the test to correctly identify pregnant buffalo) ranged from 23% on day 23–98% on day 28 post AI; the specificity (ability to correctly identify non-pregnant buffaloes) was 100% throughout the sampling period. PAG progressively decreased from parturition to day 25 post-partum; from day 30 post-partum, the concentrations fell below 1 ng/mL and were close to 0 on the last day of observation (50 d post-partum).

In conclusion, our results showed that RIA-860 is highly accurate for diagnosing pregnancy in buffaloes starting from day 28 of gestation. Furthermore, the rapid disappearance of PAG concentration after calving means that a cut-off limit in post-partum for detecting a new pregnancy is not required.

1. Introduction

Characterized for the first time in the early eighties, pregnancy-associated glycoproteins (PAG; also called pregnancy-specific protein B (PSPB) or pregnancy-specific protein 60) constitute a large family of glycoproteins expressed in the outer epithelial layer (chorion/trophectoderm) of the placenta in eutherian species

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[1–3]. PAGs are synthesized by mononucleate and binucleate trophoblastic cells, some of which are secreted into the maternal blood stream from the moment the conceptus becomes more closely attached to the uterine wall and placentome formation begins [4,5]. The accumulation of PAG/PSPB molecules in the maternal blood of ruminant ungulates allowed for the development of the radioimmunoassay (RIA) [6,7] and ELISA techniques [8] and became a useful tool for monitoring pregnancy in ruminant species.

Using different chromatographic procedures, some members of the PAG family have been isolated from the cotyledons of buffalo species [9,10] and other species from Order Cetardiodactyla [1,11–18]. Purified and semi-purified preparations have been used to immunize rabbits in order to obtain antisera (AS), which has led to the development of homologous [19] and heterologous RIA [19,20]. The first RIA system adopted for detecting PAG molecules in buffalo species was RIA-706, which uses antisera raised against caprine PAGs ($caPAG_{55kDa+62kDa}$) and purified bovine PAG as tracer [21–23].

More recently, the isolation and purification of PAGs from buffalo placenta allowed for the development of a specific RIA system for buffalo [10]. Three polyclonal antisera (AS#858, AS#859 and AS#860) were obtained against distinct buffalo PAG fractions ($wbPAG_{76kDa\_D}$, $wbPAG_{65kDa\_E}$ and $wbPAG_{58kDa}$). The highest dilution of primary antiserum (1:840,000) was obtained with AS#860, allowing distinguishing quantitative differences in buffalo PAG concentrations [10].

The aim of this study was to describe the plasma PAG profiles of water buffalo during pregnancy and postpartum periods using RIA-860 (antisera raised against PAG molecules purified from buffalo placenta). The accuracy, sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of pregnancy diagnosis for early pregnancy as well as the half-life of PAGs during the postpartum period were also described.

2. Materials and methods

2.1. Animals and experimental design

The study was carried out at the experimental farm of the Animal Production Research Centre (CREA-PCM) of Monterotondo (Rome, Italy, 42° N parallel). Ninety-eight animals belonging to a Mediterranean buffalo herd subjected to a synchronization and artificial insemination (AI) program were enrolled in the study and divided into groups as described below. Before estrus synchronization and artificial insemination program, buffalo cows were subjected to routinely veterinary clinical examination in order to exclude animals with diseases such as endometritis, mastitis and metabolic disorders. The animals enrolled in the study were at 97 ± 32.2 (mean ± SD) days in milk. The buffaloes were synchronized with a progesterone releasing intravaginal device (PRID; Sanofi, France) containing 1.55 g natural progesterone inserted *in situ* for 10 days. On day 7, an i.m. injection of 1000 IU of Pregnant Mare Serum Gonadotrophin (PMSG; Ciclogonina, Fort Dodge, Italy) and 0.15 mg of cloprostenol (PGF2α analogue; Dalmazin, Fatro, Italy) was administered. On day 10, the PRID was removed and the cows were artificially inseminated at 72 and 96 h from device withdrawal. The day of the second AI was considered as day zero.

Blood samples were taken from the jugular vein in 10 mL EDTA tubes during pregnancy on days 0 (0d), 23 (23d), 25 (25d), 28 (28d), 30 (30d) and then biweekly until the end of pregnancy. Blood samples were suspended for non-pregnant cows on day 45. The blood of 20 buffaloes that had calved was tested every five days from the day of calving until day 50 post-calving in order to determine PAG disappearance. Plasma was immediately separated by centrifugation (1200 × *g* for 15 min at 5 °C) and stored at −20 °C until assayed.

The cows involved in this experiment were treated in compliance with the animal testing regulations established under Italian law. The experimental design was carried out according to good veterinary practices under farm conditions. The CREA-PCM is authorized to use farm animals for experimental design (as stated in DM 26/96-4 of Italian Welfare Ministry).

2.2. Pregnancy diagnosis

The animals were classified as pregnant (P group) and non-pregnant (NP group) by ultrasonography and by determining plasma PAG concentrations.

Pregnancy was diagnosed on days 28 and 45 from AI by transrectal ultrasonography (Aloka–SSD Prosound 2 scanner, Hitachi Medical System, Italy) with a 7.5 MHz linear-array transducer. The same operator performed all of the ultrasound scans. Positive pregnancy status on day 28 and day 45 was characterized by the presence of embryonic vesicles and embryo proper within the embryonic vesicle and heartbeat visualization (embryo viability) [24]. In the absence of embryonic vesicle or embryo proper in the uterine lumen on day 28 and day 45, the females were considered as non-pregnant. Pregnancy status was confirmed by transrectal palpation from day 60 onwards. All pregnant buffaloes that have given birth were used as pregnant group.

Based on the PAG assay (RIA-860), a cut-off value of 1.0 ng/mL was used to distinguish between the pregnant and non-pregnant females [25]. The results of PAG RIA systems were categorised as follows: diagnosis pregnant correct (*a*); diagnosis pregnant incorrect (*b*); diagnosis not pregnant correct (*c*), and diagnosis not pregnant incorrect (*d*). From these values, the sensitivity ($100 \times a/a + d$), the specificity ($100 \times c/c + b$), the accuracy ($100 \times (a + c)/(a + c + b + d)$), the PPV ($100 \times a/a + b$) and the NPV ($100 \times c/c + d$) of the pregnancy diagnosis were calculated [26].

2.3. PAG radioimmunoassay

RIA-860 obtained with the method previously described by Barbato et al. [10] was used to measure PAG concentrations. Pure $boPAG_{67kDa}$ preparation was used as standard and tracer for all PAG assays. Iodination ($Na\text{-}I^{125}$, Amersham Pharmacia Biotech, Uppsala, Sweden) was carried out according to the Chloramine T method previously described by Greenwood et al. [27]. Primary antiserum (AS#860) was used at an initial dilution of 1/840,000 (AS#860).

In short, firstly the samples were essayed in a preincubated system in which the standard curve ranged from 0.2 to 25 ng/mL. Samples with higher PAG concentrations than the estimated standard dose at which the percentage B/B_0 was 20% (ED_{20}) were re-assayed in non-preincubated systems in which the standard curves ranged from 0.8 to 100 ng/mL.

The minimum detection limit (MDL), calculated as the mean concentration minus twice the standard deviation (mean − 2 SD) of 20 duplicates of the zero (B_0) standard [28], was 0.4 ng/mL.

2.4. Statistical analysis

Statistical analysis were performed with SPSS 23.0 (SPSS Inc. Chicago, USA) and considered as significant at a level of 0.05.

Data were analysed by Linear Mixed models. “Time” (days post AI or post-partum) was included in the model as repeated measure with a scaled identity covariance structure and Buffalo as random factor. RIA-860 concentrations were analysed during early pregnancy in order to investigate differences between the pregnant and non-pregnant buffalo cows. These models evaluated the effects of