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

O. Barbato[a], *, L. Menchetti[a], N.M. Sousa[b], A. Malfatti[c], G. Brecchia[a], C. Canali[a], J.F. Beckers[b], V.L. Barile[d]

a Department of Veterinary Medicine, University of Perugia, 06126, Italy

b Laboratory of Animal Endocrinology and Reproduction, Faculty of Veterinary Medicine, University of Liege, 4000, Belgium

c Department of Veterinary Science, University of Camerino, 62032, Italy

d Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA), Animal Production Research Centre (CREA-PCM), Monterotondo, Rome 00015, Italy

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(xþ1) 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 23e98% 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.

Keywords: Buffalo, Pregnancy-associated glycoprotein Radioimmunoassay, Pregnancy Postpartum

1. Introduction

Characterized for the first time in the early eighties, pregnancy- associated glycoproteins (PAG; also called pregnancy-specific pro- tein 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[1e3]. 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 develop- ment 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 [1,11e18]. 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 (caPAG55kDaþ62kDa) and purified bovine PAG as tracer [21e23].

More recently, the isolation and purification of PAGs from buf- falo 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 (wbPAG76kDa_D, wbPAG65kDa_E and wbPAG58kDa). The highest dilu- tion 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

2.1Animals 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 synchroni- zation 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 synchro- nized 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 (PGF2a 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 compli- ance 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 trans- rectal ultrasonography (AlokaeSSD 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 _ a/ a þ d), the specificity (100 x c/c þ b), the accuracy (100 x (a þ c)/ (a þ c þ b þ d)), the PPV (100 x a/a þ b) and the NPV (100 x c/c þ d) of the pregnancy diagnosis were calculated [26].

2.3. Hormone and metabolite assays

RIA-860 obtained with the method previously described by Barbato et al. [10] was used to measure PAG concentrations. Pure boPAG67kDa preparationwas used as standard and tracer for all PAG assays. Iodination (Na-I125, 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/B0 was 20% (ED20) were reassayed 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 e 2 SD) of 20 duplicates of the zero (B0) standard [28], was 0.4 ng/mL.

2.4. Statistical considerations and data 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 Time (6 levels: 0, 23, 25, 28, 30, and 45 days post AI), Outcome of AI (2 levels: pregnant and non-pregnant), and interaction Time x Outcome of AI. Moreover, following the categorization of the RIA-860 concentrations according to the threshold of 1.0 ng/mL (PAG < 1.0 ng/ mL ¼ Non- Pregnant; PAG 1.0 ng/mL ¼ Pregnant), accuracy, sensitivity, specificity, PPV and NPV were calculated on days 23, 25, and 28 post AI as stated above. Twenty-four time points during pregnancy (0, 23, 25, 28, 30 and biweekly samples taken after 30 days until the end of pregnancy) and 11 after calving (every 5 days from parturition to day 50 postpartum) were included in Linear Mixed models in order to investigate the profile of PAG concentrations during pregnancy and the post-partum period. Effects of Time were evaluated. Sidak corrections were used to pairwise comparisons. Diagnostic graphics were used to check assumptions and outliers and Log(xþ1) transformation. Results were expressed as back-transformed estimated marginal means ± standard error (SE) while row data were presented in figures. Lastly, the elimination rate constant was calculated from the slope of the line during the post-partum period by linear regression analysis of the semilogarithmic plot of PAG concentrations versus time while the half-life was obtain as (ln 2/elimination rate constant).

3. Results

Fifty-two out of the 98 buffalo cows enrolled in this study became pregnant (P group) while 46 remained non-pregnant (NP group) as determined by ultrasonography and PAG RIA analysis.

3.1. PAG-860 concentrations in pregnant and non-pregnant buffaloes until day 45 post AI

Until day 45 post AI the PAG concentrations were affected by Time (P < 0.001), Outcome (P < 0.001), and Outcome x Time effect (P < 0.001). As shown in Table 1, the mean concentrations of the P group measured with RIA-860 progressively increased from day 23 (0.3 ± 0.0 ng/mL; P < 0.01) until day 45 (11.7 ± 0.0 ng/mL; P < 0.0001), while they remained constantly close to zero for the NP group.

Pairwise comparisons showed that there were significant differences between the P and the NP groups from day 23 post AI (mean difference: 0.3 ± 0.1 ng/mL; $P < 0.001$) until day 45 of observation (mean difference: 11.7 ± 0.1 ng/mL; $P < 0.001$; Table 1). On day 28, only one female in the P group exhibited PAG concentrations below 1.0 ng/mL. On day 30 PAG concentration 1.0 ng/mL were detected in all pregnant animals. The accuracy and sensitivity of the pregnancy diagnosis increased from day 23 to day 28, as shown in Table 2.

3.2. Profile of PAG-860 concentrations during pregnancy and postpartum

The PAG concentrations were affected by Time ($P < 0.001$) during pregnancy and the postpartum period. The mean RIA-860 concentrations increased progressively reaching higher values than on the day of insemination (0.1 ± 0.1 ng/mL) throughout the gestational period and peaked on day 240 post-insemination (29.9 ± 0.1 ng/mL). However, the PAG concentrations stabilized after day 105 post-insemination as no significant differences were observed from day 105 post-insemination (20.5 ± 0.1 ng/mL; $P < 0.05$) until the end of pregnancy (26.0 ± 0.1 ng/mL; Fig. 1). The PAG concentrations decreased progressively from parturition (42.1 ± 0.1 ng/mL) until 35 days post-partum (0.3 ± 0.1 ng/mL; $P < 0.001$) reaching a value below 1.0 ng/mL 30 days post-partum. They remained close to zero on the last day of observation (50 d post-partum: 0.0 ± 0.1 ng/mL; Fig. 2). Kinetic parameters were calculated by scatter plots of ln RIA- 860 concentrations versus post-partum days. The elimination rate constant was 0.12 d1 while the half-life was 5.8.

4. Discussion

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

In the first period of gestation, significant differences were observed in the PAG profile of pregnant and non-pregnant buffaloes from day 23 onwards.

Karen et al. [21] studied the PAGs of buffalo from day 19 to day 55 after mating, using caprine PAG (RIA 706). It proved to be an accurate test for detecting pregnant buffaloes in the time window from days 31e35. With the same RIA system between day 28 and 103 of pregnancy, El-Battawy et al. [22] observed great differences in the PAG concentrations of pregnant and non-pregnant buffaloes from day 28 onward. In our previous study [23], an increase in the PAG concentration of pregnant buffaloes was observed starting from week 6 of gestation, using RIA-706. This study shows that using RIA-860 improves the accuracy of detecting pregnant buffaloes on day 25 and reaches 99% on day 28. However, due to the high individual variability in the PAG concentration, the PAG values cannot be utilized to predict the pregnancy-day.

During the gestation period, PAG concentration increased up to day 105 and then remained constant up to parturition. This trend was similar to those previously described in buffalo [23], and to that reported for Zebu cattle (Bos indicus) [16] but differed greatly to the trend observed for bovine (Bos taurus), for which an exponential increase was observed until parturition [19].

The buffalo PAG pregnancy profile showed more similarities with that of some wild ruminant species such as reindeer [29] and withe-tailed deer [30], although the concentrations observed for these species appear lowest than those of buffalo. A similar buffalo PAG profile was recorded for goats by Sousa et al. [16] even if Gonzalez et al. [31] observed an increase for the same species between the sixth and the tenthweek of pregnancy. Similar pattern to that found in our workwas also observed in various breeds of sheep [32], but differs from those described by Ranilla et al. [33,34], who after an initial increase, observed a return to baseline levels around mid-pregnancy.

At parturition buffalo cows reached lower mean PAG concentrations (42.1 ng/mL) than dairy cows (2462.4 ng/mL) [19] or zebu cows (1095 ng/mL) [35].

In the post-partum period, PAG concentrations decrease rapidly reaching minimum values (<1 ng/mL) on day 30. These data differ from those reported by using three heterologous PAG RIA systems [23] in which the PAG concentration decreased gradually until week 8 postpartum, when the minimum value (<1 ng/mL) was registered.

A comparable decline in PAG concentrations in the first month of postpartum period was reported for ewes [33,34], goats [16,31] and for wild ruminants [29,30]. In these species, the PAG concentration reaches levels lower than 1 ng/mL on day 30 postpartum. Contrastingly, PAG concentration decreases slowly in cows following parturition and could be detected as late as day 100 postpartum [19]. The rapid decrease in PAG concentration during the post-partum period is essential when using PAG as an appropriate marker of pregnancy just after calving. Unlike bovines, the rapid PAG disappearance in buffalo does not require a cut-off limit in post-partum animals as a means for detecting a new pregnancy, as there is a voluntary waiting period of at least 50 days.

The half-life proved to be shorter in buffaloes (5.8 day) than in cattle (from 7.0 to 8.8 days) [35e37], goats (7.5 day) [38] and in Zebu (9.2e10.1 days) [35], yet it was longer than the half-life of sheep (4.5) [38]. These differences could be due to the presence of N-linked carbohydrate and sialic acid chains on their structure [35,39].

In conclusion, for the first time in literature this study showed the plasmatic profile of PAG in buffalo during gestation and the post-partum period using homologous antisera. On day 28 post AI, the percentages of accuracy (99%), sensitivity (98%), and specificity (100%) were almost identical, thus proving that RIA-860 may be a highly accurate test for diagnosing pregnancy in buffaloes starting from day 28 of gestation. Furthermore, the rapid disappearance of PAG concentration following calving, does not require a cut-off limit in post-partum for detecting a new pregnancy.

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Fig. 1. PAG concentrations during pregnancy in water buffaloes (mean ± SE, row data) evaluated with RIA-860. The values with asterisks are significantly different (***P < 0.001, **P < 0.01) to those of the previous day.

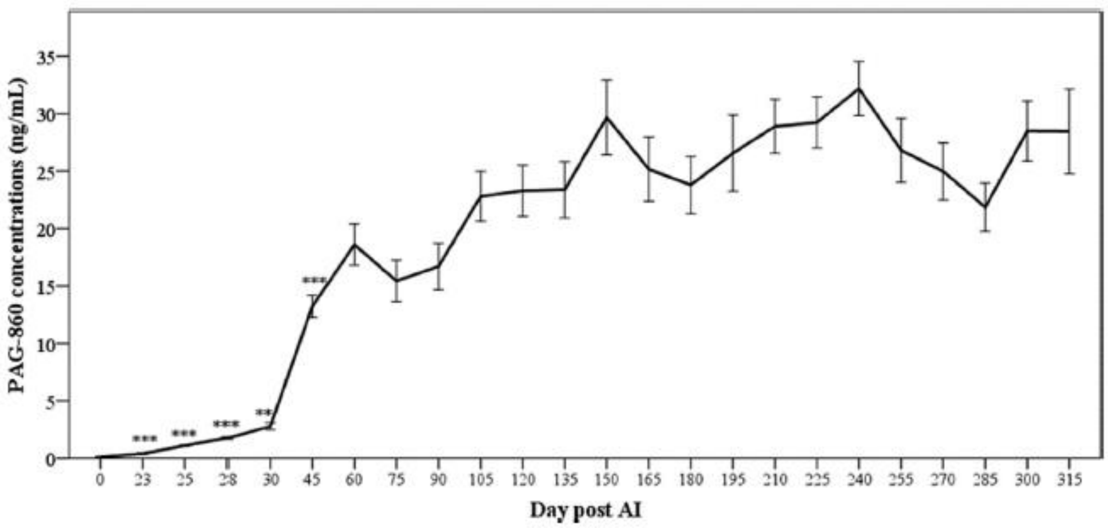

Fig. 2. PAG concentrations of water buffaloes during the post-partum period (mean ± SE) evaluated with RIA-860. The values with asterisks are significantly different (***P < 0.001, **P < 0.01, *P < 0.05) to those of the previous day.

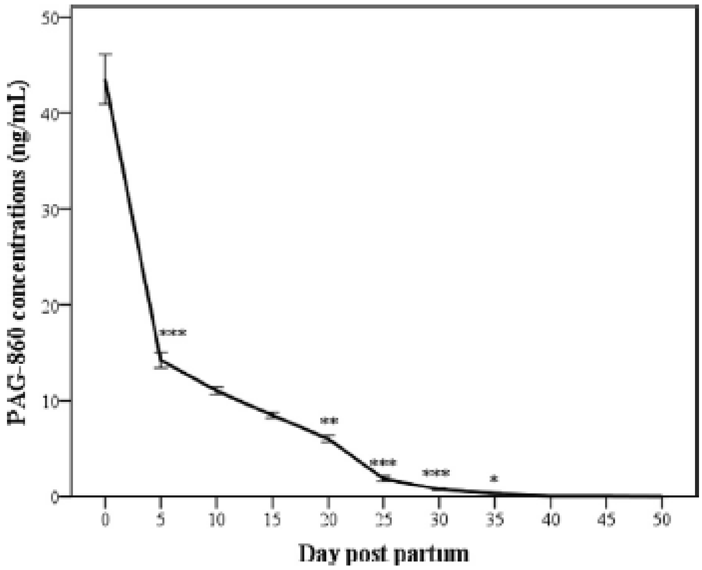

Table 1

PAG concentrations (ng/mL) in non-pregnant (NP, n ¼ 46) and pregnant (P, n ¼ 52) water buffaloes (mean ± SE) until day 45 post AI (back-transformed estimated marginal means ± SE).

Group	Day post AI						P value[a]
	0	23	25	28	30	45	
NP	$0.0_a \pm 0.0$	$0.0_a \pm 0.0$	$0.1_a \pm 0.0$	$0.0_a \pm 0.0$	$0.0_a \pm 0.0$	$0.0_a \pm 0.0$	0.953
P	$0.1_a \pm 0.0$	$0.3_b \pm 0.0$	$1.1_c \pm 0.0$	$1.6_d \pm 0.0$	$2.5_e \pm 0.0$	$11.7_f \pm 0.0$	0.000

[a] Univariate tests. Values followed by the same letter in each row do not differ significantly (P < 0.05; Sidak's multiple comparison test).

Table 2

Sensitivity, specificity, accuracy, positive predictive value (PPV), and negative predictive value (NPV) of RIA- 860 systems for pregnancy diagnosis in 98 water buffaloes 23, 25, and 28 days post-insemination.

Day	Diagnosis	Outcome[a]		Sensitivity	Specificity	Accuracy	PPV	NPV
		Non pregnant (n = 46)	Pregnant (n = 52)					
23	Non pregnant	46 (100.0%)	40 (76.9%)	23	100	59	100	53
	Pregnant	0 (0.0%)	12 (23.1%)					
25	Non pregnant	45 (97.8%)	12 (23.1%)	77	98	87	98	79
	Pregnant	1 (2.2%)	40 (76.9%)					
28	Non pregnant	46 (100.0%)	1 (1.9%)	98	100	99	100	98
	Pregnant	0 (0.0%)	51 (98.1%)					

[a] Evaluated by ultrasonography.