JOURNAL OF PHYSIOLOGY AND PHARMACOLOGY 2006, 57, Supp 8, 153–171
www.jpp.krakow.pl

N.M. SOUSA[1], A. AYAD[1], J.F. BECKERS[1], Z. GAJEWSKI[2]

PREGNANCY-ASSOCIATED GLYCOPROTEINS (PAG) AS PREGNANCY MARKERS IN THE RUMINANTS

[1]Physiology of Animal Reproduction, Faculty of Veterinary Medicine, University of Liege, B-4000, Belgium, [2]Department of Animal Reproduction, Faculty of Veterinary Medicine, WAU, Warsaw, Poland

In the last years, a polymorphic family of placenta-expressed proteins has been discovered in ruminant species and used for pregnancy diagnosis. Pregnancy diagnosis is an important part in reproduction management of ruminants. The pregnancy-associated glycoproteins (PAG) are synthesized in the mono- and binucleate cells of the ruminant's trophectoderm. Part of them is released into maternal blood circulation where they can be assayed by different RIA and ELISA systems. Due to large variety of expressed molecules and to large variations in the post-translational processing of the glycoproteins, different immuno-systems present different ability to quantify the PAG released in blood. Recent investigations showed that surprisingly the level of milk production in ruminants can modify the concentration of PAG circulating in blood. On the whole, the data show that the RIA methods are very precise for measuring PAG concentrations in the maternal blood and milk of the ruminants. Different studies clearly indicate that milk can be used for pregnancy diagnosis in small ruminants. The sensitivity and specifity of this method are very high. The results showed the possibility of the use PAG in milk and in blood as pregnancy test. It is especially helpful in the diagnosis of gestation and in detection of embryonic mortality as a non stressed method in the pregnancy management in the ruminants.

Key words: *pregnancy-associated glycoproteins, pregnancy diagnosis, ruminant, markers*

STRUCTURAL AND BIOCHEMICAL GENERALITIES CONCERNING THE EUTHERIAN PREGNANCY-ASSOCIATED GLYCOPROTEIN (PAG) FAMILY

Pregnancy-associated glycoproteins, (PAG) also known under a variety of other names including pregnancy-specific protein B (PSPB) (1, 2), pregnancy-

specific protein 60 (PSP-60) (3) and SBU-3 antigen (4), were first described as placental antigens that were also present in the blood serum of the mother soon after implantation. Characterized in the last 25 years (1, 5 - 8), they constitute a large family of glycoproteins expressed in the outer epithelial cell layer (chorion/trophectoderm) of the placenta of eutherian species. They are synthesized by the mono- and binucleate thophoblastic cells, some of them being secreted in maternal blood from the moment when the conceptus becomes more closely attached to the uterine wall and formation of placentomes begins (9). By using biochemical procedures, some molecules of the PAG family were isolated from cotyledons of cow (7, 10, 11), ewe (12 - 14), goat (15), buffalo (16), bison (17), moose and elk (18). Purified and semi-purified preparations were used to immunize rabbits and the antisera obtained allowed the development of homologous (3, 19 - 22) and heterologous radioimmunoassay (RIA) (23 - 25) and sandwich enzyme-linked immunosorbant assay (ELISA) systems (26). Screening of placental libraries with nucleic acid probes has identified additional (probably more than 100) cDNA that are very abundant and code for polypeptides related but generally distinct from PAG isolated by biochemical procedures (12-15, 27, 28). Recent investigations have also demonstrated that different PAG cDNA are not expressed coordinately throughout pregnancy (27 - 29). Some, for example, are expressed early, others only when pregnancy progresses.

An important feature of ruminant PAG is that they are extensively glycosylated proteins undergoing a complex posttranslational processing, with the carbohydrate signature of the trophoblastic cells (30). Apparent molecular masses of purified PAG showed a major variability, having higher estimated values (55 to 70 kDa) than the expected molecular mass of their protein core (37 kDa) (11, 31). The variable degree of glycosylation in the different PAG has been claimed to be an important factor regulating plasma half life of these proteins including their peripheral concentration (11). A similar situation exists in primates and equids with the existence of the more or less glysolated forms of the human chorionic gonadotrophin (hCG) and the equine chorionic gonadotrophin (eCG).

It is also important to remember that the PAG molecules belong to a group of proteolytic enzymes known as aspartic proteinases (AP) (8, 32), having more than 50% amino acid sequence identity with pepsin, cathepsin D and cathepsin E. Comparative molecular modeling studies have demonstrated that PAG have retained the well-known bilobed structure of AP, containing a peptide binding cleft between the two lobes capable of accommodating peptides up to 7 amino acids (33). In spite of the fact that most PAG are assumed to be enzymatically inactive due to key mutations within their binding cleft, some reports (33, 34) have claimed that they can bind to pepstatin A (a 6 amino acid peptide), which is an extremely powerful inhibitor of AP. It remains to be determined whether the PAG expressed in the ungulate placenta are catalytically active and, even if they are, whether their physiological main function is that of proteinases.

THE BOVINE PAG FAMILY

After the isolation and the characterization of the first PAG in bovine placenta (known as boPAG–1 or boPAG$_{67kDa}$) (7, 8), different PAG molecules were identified, justifying an arbitrary numbered nomenclature adopted by different authors. Till now, complementary DNA (cDNA) of the 22 boPAG genes (boPAG–1 to boPAG–22) were cloned and fully sequenced in cows (8, 27, 32, 35, 36). Recently, PAG molecules were classified into three main groups, according to both their amino acid identity and their phylogenetic linkages: the boPAG–8, the boPAG–2 and the boPAG–1 groups. Hypothesis on the physiological role of these three main PAG subfamilies, as well as their utilisation for pregnancy diagnosis in veterinary practice will be described in the following paragraphs.

The pepsinogen-like PAG (boPAG–8) group

Bovine PAG–8 group is possibly the most ancient so far identified (35). Close related proteins were described in Perissodactyla (horse and zebra PAG) (37), Carnivora (cat PAG) (38), Lagomorpha (rabbit pepsinogen F) (39) and Rodentia (mouse and rat pepsinogens F) (40, 41), as well as in bovine (boPAG–10) and other ruminant species (e.g. caprine PAG–8 and porcine PAG–8). It has been hypothesized that boPAG–8, as well pepsinogen F, are enzymatically active (42). In fact, despite the great economical interest of chymosin and pepsinogen –largely used in cheese industry–, the members of the aspartic proteinases family expressed during the prenatal life of the bovine are not yet studied; the molecules were not isolated starting from fetuses digestive tracts. This work is in progress in our laboratory. However, till now, due to the inexistence of a purified boPAG–8 preparation, no clinical investigations could be carried out in order to elucidate the plasmatic profiles and hypothetical role of these proteins during pregnancy.

The gonadotropin-like PAG (boPAG–2) group

As early as in 1940, special cells of the ruminant placenta were suspected as responsible for an important endocrine function during gestation. In this decade, it was observed for the first time a reduction in the pituitary gonadotropic activity in pregnant cows and suggested that the corpus luteum was supported by additional luteotropic substances produced elsewhere. Using microsections of cotyledons and staining for carbohydrates with periodic acid-Schiff (PAS), Weeth and Herman (43) and Björkman (44) described the presence of numerous trophoblastic cells containing glycoproteins. About 10 years later, Foote and Kaushik demonstrated the presence of a LH-like activity in the cotyledons (45). They used the bioassay of Parlow in order to identify this LH-like activity (46). These studies were pioneers in this field: they opened the way for further identification and characterization of placental hormones and glycoproteins in the ruminant species.

The research of a chorionic gonadotrophin in bovine placenta (bCG) continued with Ailenberg and Shemesh who looked for a gonadotrophic-like substance capable to stimulate the progesterone production in bovine granulosa cells culture (47). For this purpose, they used a radio-receptor method on Leydic cells according to the technique previously described by Ramachandran and Sairam (48). They isolated a thermolabile substance with an apparent molecular mass of 60 kDa and with a biological activity related to that of bovine LH. They considered this factor as a true bovine chorionic hormone. This hormone could have a special importance in the cow, as in this species, the corpus luteum is required for the maintenance of gestation throughout most of this process (at least for 200 days).

In 1987, Beckers et al. continued to study this question by using the radioreceptor assay method with membrane receptors and by looking for a luteotrophic activity in fetal cotyledonary extracts and in all the fractions obtained during biochemical purification procedures (49). The membrane receptors were prepared from corpora lutea after tissue homogenization followed by centrifugation. After several successive purification steps, they isolated a substance with an activity 400 times greater than that isolated by Ailenberg and Shemesh (47). The molecular mass of this bCG was 30 kDa. This hormone could not be confounded with hypophyseal LH as there was no arc of precipitation between purified bCG and anti-bLH antiserum following double radial immunodiffusion (DID) in agarose gel. However, this purified hormone was closely related to pituitary LH as the anti-bCG serum raised in rabbit presented a cross-reaction with bovine and ovine LH in DID.

The question on the existence of a gonadotrophic substance in ruminant placenta was partly solved by the study of Xie *et al*. (32), who showed that the cDNA sequence corresponding to the bCG molecule was closely related to those of the aspartic proteinase family. This data meaning that aspartic proteinase could stimulate the luteal function was highly surprising. However, previous investigations have shown that chymotrypsinogen, a serine proteinase, can bind to the LH/hCG receptor and activate it (50).

One other answer to this important question could be given by the investigation described by Nilson *et al*. (51). These authors showed that the expression of the chorionic gonadotrophin α-subunit gene occurs in the pituitary of all mammals, but only in placenta from primates and horses, and not in other species. Although this previous study needs confirmation by other complementary investigations, it comforts the hypothesis of a certain luteotropin-like activity of the bovine pregnancy-associated glycoprotein 2 (boPAG–2) (52).

The boPAG–2 is a polypeptide of 372 amino acids long, structurally related to boPAG –11, –12 and –13 (62%, 83% and 96% amino acid sequence identity, respectively), as well as with PAG–2 from ovine and caprine origins (64% and 63% amino acid sequence identity, respectively) (32). As described by Xie *et al*., boPAG–2 is synthesized by placental explants as a 70 kDa molecular mass

protein that is processed to smaller molecules (32). Expression of boPAG–2 mRNA is detected as early as 17-19 days of pregnancy, coinciding with the beginning of implantation. Its mRNA is expressed in fetal placenta but not in other fetal organs, and is localized in both mononucleate and binucleate cells. Unfortunately, as no highly purified boPAG–2 preparation is available, no specific RIA system could be developed in order to detect boPAG–2 during early pregnancy.

The major bovine PAG family, the boPAG–1 group:

Several close related PAG molecules were identified from the time of early blastocyst development (53, 54) until parturition (21, 30). The first PAG purified from bovine fetal cotyledons was named boPAG–1 or $boPAG_{67kDa}$ (7). Recently, it was demonstrated that lectins such as *Vicia villosa* agglutinin (VVA) and *Dolichos biflorus* agglutinin (DBA) bind to N-acetylgalactosamine (GalNAc) of asparagines-linked glycans on bovine PAG (31). By the use of this technique, Klisch *et al*. isolated 3 distinct PAG molecules from bovine cotyledons: $boPAG_{56kDa}$, $boPAG_{67kDa}$ and $boPAG_{75kDa}$ (11). Although, some effort was done in order to purify new PAG molecules, till now, the number of purified proteins belonging to the boPAG–1 group remains much lower than the number of identified cDNA.

Immunocytochemical and in situ hybridization investigations allowed the localization of PAG–1 molecules predominantly in the cytoplasm of binucleate cells present in the fetal cotyledonary tissue (27, 55). Based on their cell specificity and on their sequences, Hughes *et al.* demonstrated that the divergence time between the trophoblast binucleate cell-expressed genes (PAG–1) and the PAG genes expressed throughout the trophectoderm (PAG–2) is estimated to be approximately 87 million years ago (29). Interestingly, the presence of antigens immunologically similar to $boPAG_{67kDa}$ has also been demonstrated in testicular tissue and in ovarian extracts justifying the adjective “associated” and not “specific” given to this glycoprotein (56, 57). However, at our best knowledge, no further investigation was made in this way and so, the hypothetical synthesis of a molecule related to PAG in the Sertoli cells and ovarian tissue was not confirmed experimentally. No precise function could be experimentally demonstrated for molecules from boPAG–1 group (58, 59). However, their high level of expression in early gestation (54, 60) point to a fundamental role of such as molecules in implantation and placentogenesis (61). Recently, by use of cDNA microarray analysis, Ushizawa *et al*. demonstrated that several PAG molecules are expressed as early as Day 7 to 14 of pregnancy (boPAG–11, –16, –17), Days 14 to 21 (boPAG–1, –5 to –7, –9 to –13, –15 to –17, –19, –21) or even before (at Day 7: e.g. boPAG–4, –5 and –6) (54). However, as most of these proteins could not be purified, they are not yet available for RIA and ELISA specific measurements.

The classical PAG-497 radioimmunoassay

In 1992, Zoli *et al.* described the validation of a homologous PAG RIA with the use of boPAG$_{67kDa}$ as standard, tracer and immunogen for antiserum production (AS#497) (21). By use of this RIA (RIA-497), PAG can be detected in maternal circulation of some cows at around Day 28 of pregnancy, and in all cows (concentrations higher than 0.5-0.8 ng/ml) from 30 to 35 Days of pregnancy. In early and mid gestation, concentrations increase slowly and gradually, remaining below than 160 ng/ml till Day 240 of pregnancy. Around parturition, PAG concentrations increase rapidly to reach peak values of 1 to 5 µg/ml only few days before delivery (21). Concentrations decrease steadily in the postpartum period reaching undetectable levels only by day 100 postpartum (21). The relatively long time needed for PAG to be cleared from maternal circulation can be explained by the very high concentrations present in maternal blood at parturition and by a long half-life of this glycoprotein, estimated to be 7.4 to 9 days (62 - 64). Investigations realized in peripartum clearly demonstrated the positive influence of both maternal environment and fetal genotype (sex and race) on peripheral blood concentrations of bovine PAG molecules (21, 65, 66). Higher PAG concentrations are observed in maternal than in fetal serum, suggesting that this glycoprotein is delivered preferentially in the maternal system (21).

Alternative PAG radioimmunoassays

Interest in improving existing immunoassay methods for PAG by testing new antisera (25, 67, 68) can be explained by different phenomena such as the temporal expression of different PAG molecules during early pregnancy, the high N-terminal amino acid identities and distinct glycosylation patterns (and probably half-life) of PAG molecules purified from ovine, caprine or bovine placenta. These parameters can explain the specific ability of different antisera to detect PAG during early pregnancy in cattle (25, 66, 69, 70).

Recently, Perényi *et al*. compared anti-boPAG$_{67kDa}$ antiserum with new antisera raised against PAG molecules isolated from caprine placenta (PAG$_{55kDa+62kDa}$ and PAG$_{55kDa+59kDa}$, AS#706 and AS#708, respectively) (68). Interestingly, despite the use of the same boPAG$_{67kDa}$ preparation as standard and tracer, the new developed systems (RIA-706 and RIA-708) revealed much higher PAG concentrations between Days 25 and 50 after AI, suggesting that epitopes from PAG molecules earlier expressed can be better recognized by antisera raised against PAG coming from other species closely related to the bovine. In the same way, in a preliminary study, we demonstrated that the use of pooled antisera (a mixture of 4 rabbit antisera against PAG from bovine (AS#497), ovine (AS#780 and AS#809) and caprine (AS#706) origins) can be useful for routine pregnancy diagnosis in bovine species (25). The use of this pool increases the distance between the values found in two different population: non pregnant females and pregnant at day 30 after AI.

An original approach was recently developed by Lopez-Gatius *et al.*, who determined plasma levels of boPAG (measured by both RIA-497 and RIA-706) and progesterone in Holstein Friesian dairy cows (70). Cows were followed by venipuncture on Days 35, 42, 49, 56 and 63. The results showed an interaction between the milk production and the PAG levels measured by both RIA systems. The effect was more marked using the AS#497. The concentrations of PAG decreased when dairy milk production increased whereas the progesterone levels remained unaffected by milk production. It remains to be determined if the PAG decrease is due to an increased traffic of PAG to the mammary gland or to an increased clearance due to a higher metabolic activity.

Based on PAG determinations (RIA-497, RIA-706 and RIA-708) and ultrasound fetal measurement, Chavatte-Palmer et al. demonstrated that cattle recipients carrying somatic clones showed alterations in development speed (69). However, further longitudinal studies with sequential measurement of different placental proteins (PAG by different RIA systems, placental lactogen, etc) are necessary to clarify the chronology of trophoblastic alterations of placentas issued from nuclear transfer techniques or embryos cultured in atypical conditions… In different studies, we could follow progesterone and PAG concentrations in females (cows and heifers) having received an embryo at day 7 after the reference estrus. Generally, the recipients are transferred if the reference estrus appeared clearly and if the corpus luteum can be identified on the ovary. The embryo is transferred in the ipsilateral horn of the uterus. A retrospective analysis of the profiles after embryo transfer reveals an atypical case (*Fig. 1*). The recipient R599 was transferred without an active corpus luteum as revealed by the low P4 concentrations at any time of the venipuncture. We can hypothesize that this recipient had probably a low ovarian activity (low estrogen levels) allowing the survival of the embryo in the uterus. However, the lack of P4 and the low concentrations of embryotrophs substances stimulated an earlier production of PAG as revealed by the different RIA. Finally, the embryo could not survive, the PAG levels decreased and an estrus was clinically detected at day 56 after the reference estrus. Such retrospective observations are rare and the investigations are generally not completed. However, this case gives new arguments for a reciprocal “dialogue” between uterus environment and embryonic trophoblast.

PAG enzyme-linked immunosorbant assay

Recently, a ‘sandwich’ type of enzyme-linked immunosorbant assay (ELISA) was also made available and was used to detect PAG concentrations in Holstein cows and heifers. In this system, a mixture of monoclonal antibodies raised against semi-purified PAG molecules produced in early pregnancy (Days 24, 34 and 80) was coated in the wells. A polyclonal rabbit antiserum raised against PAG purified from mid-pregnancy cotyledons (Day 150) was used to bind the immobilized PAG, this complex being revealed by use of an alkaline

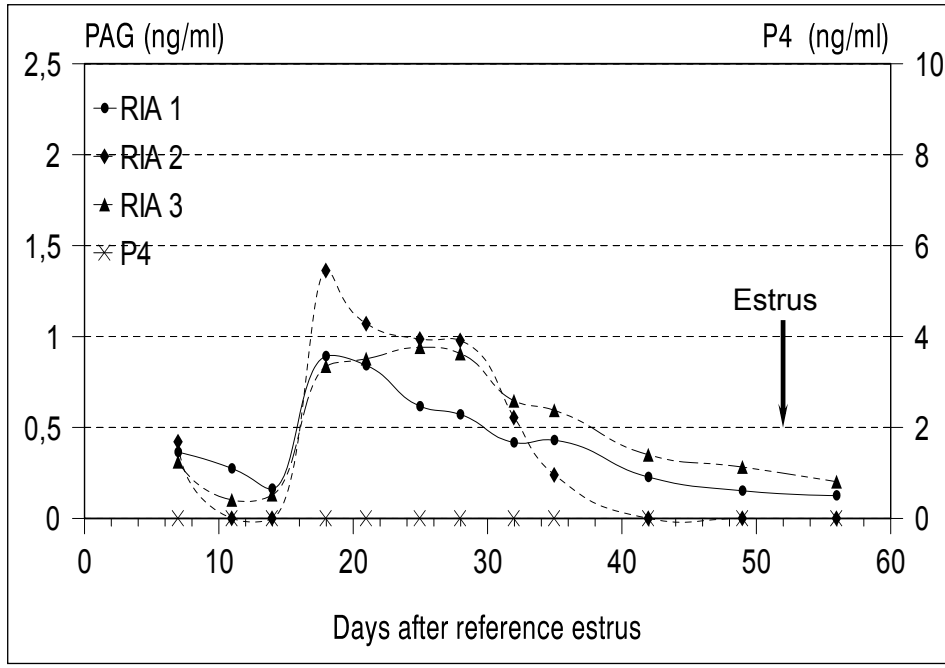

Fig. 1 During the observation period the cow n° R599 did not express detectable levels of P4, retrospectively, the concentrations of P4 indicate the lack of an active corpus luteum. This pathology, often observed in north countries in winter, was described as "Functional anoestrus". However, an embryo transfer was performed at Day 7 after the sign of the last estrus. At Day 52 new signs of estrus were recorded.
Around Day 18, a small peak was observed in the PAG concentrations indicated by RIA 1-2 (497-706) systems, and at Day 25 by RIA 3 (708) system. Thereafter, the PAG levels started to decrease till the end of the observation period. These profiles show a very early synthesis of PAG associated with a short period of expression. The embryo could not survive in the uterus due to the lack of progesterone.

phosphatase-conjuged anti-rabbit antibody. The concentrations of PAG measured by ELISA rose rapidly between Days 24 and 28 of pregnancy (26). However, the ability of this assay to discriminate open and pregnant females in early gestation was not yet analyzed.

Pregnancy diagnosis in cattle

In veterinary practice, the measurement of molecules from boPAG–1 group in peripheral maternal circulation has been used for both pregnancy confirmation and follow-up of the trophoblastic function (19, 20, 71, 72). The first aspect can help breeders in the management of reproduction (73 - 76), while the second concerns more specifically clinicians and researchers in their investigation to establish differential diagnosis of pathologies affecting pregnancy (69, 77).

Pregnancy diagnosis by RIA-497 is recommended from days 28 (73, 74) to 30 (19, 20) after breeding. However, by use of this system including AS#497, detectable levels of PAG have been also found in about 20% of virgin heifers and non-pregnant cows and 15% of bull sera (21). Alternative radioimmunoassay systems have proven to detect higher PAG concentrations as early as Day 25-30 of gestation (25). However, field studies are necessary in order to confirm their use in routine diagnosis at earlier pregnancy periods.

Still concerning routine diagnosis, an ELISA test for PSPB (BioPRYN™, BioTracking, Moscow, ID, USA) was made available being recommended for pregnancy detection from Day 30 after AI or mating in dairy and in beef cows, and from Day 28 in heifers.

THE OVINE PAG FAMILY

As early as in 1988, the identification of placental antigens immunologically related to bovine PAG/PSPB in the peripheral circulation of pregnant ewes (78) have encouraged their isolation and characterization in this species. Isolated molecules took the following names: ovine PAG (ovPAG) (79), ovine PSPB (oPSPB) (80) and SBU-3 antigen (4).

The first purified and characterized ovine PAG (designated ovPAG–1) was initially identified as a molecule closely related to the boPAG–1, containing 382 amino acids (8). Molecular cloning studies have shown that ovPAG–1, like boPAG–1, belongs to the aspartic proteinase family showing 73% amino acid sequence identity with this protein (8). Two years later, a cDNA coding for an additional member of PAG family (ovPAG–2) was identified in ovine placental library prepared from day 13 whole conceptus (81). More recently, 9 additional cDNA coding for distinct PAG (ovPAG–3 to ovPAG–11) were identified in ovine placenta at different gestational periods, confirming the multiplicity and temporal expression of PAG molecules in ruminant placenta (12, 27, 35).

As previously described for boPAG family, ovPAG–2 is expressed in both mononucleate and binucleate cells throughout the trophectoderm whereas a large number of ovPAG molecules (ovPAG–1 and ovPAG–3 to –9) are predominantly expressed in binucleate trophectoderm cells (27). Several ovPAG have been purified from conditioned media from 100 Day-ovine placental explants (12), as well as from cotyledonary tissue collected between Day 60 to 100 (14) and after Day 100 of pregnancy (13). By use of a series of chromatography procedures, a total of 15 ovPAG having molecular masses ranging from 55 to 66 kDa ($ovPAG_{55kDa}$ to $ovPAG_{66kDa}$) could be isolated and characterized (12 - 14). Another subset of PAG was identified in extracts of sheep placenta by Atkinson *et al.* (4) who used the monoclonal antibody against SBU-3 developed by Gogolin-Ewens *et al*. (82). The three proteins affinity-purified with the SBU-3 antibody had

molecular weights of 57, 62 and 69 kDa, showing partial amino acid sequence homology to the ovPAG–1, boPAG–1 and rabbit pepsinogen F (4).

The analysis of glycan (or carbohydrate) content of SBU-3/ovPAG were consistent with the major chains being sialylated and having multiple antennary N-linked chains (17.83% of the relative molecular mass of ovPAG/SBU3) (4). In these molecules, the large amounts of lateral chain sugars have been shown to be made up of N-acetyl glucosamine (5.26%), N-acetyl neuraminic acid (4.25%), N-acetyl galactosamine (3.62%), galactose (1.94%) and mannose (1.81%). As described by Xie *et al*., potential sites for glycosylation range from 2 (ovPAG-2) to 7 (ovPAG-8) in ovine species (35). However, biological implications related to glycan contents of PAG could not be demonstrated experimentally.

PAG and PSPB radioimmunoassays

During nineties, all investigations concerning PAG detection in ovine species were carried out by the use of heterologous RIA, which were based on the use of ovine (AS#495) or caprine (AS#706 and AS#708) antisera, and a bovine preparation (bPSPB or $boPAG_{67kDa}$) as standard and tracer (23, 78, 80, 83). Very recently, after achieving the purification and characterization of PAG from ovine placentas collected at different stages of pregnancy, our researches allowed the production of semi-purified preparations (13, 14) to be used as standard, tracer and immunogens for polyclonal antisera production (22). PAG can be detected in sheep maternal blood at the 3^{rd}-4^{th} week after breeding (23, 80). In a more recent investigation, by the use of two ovine-based PAG RIA systems (RIA-780 and RIA-805), we demonstrated that from day 18 onward, all non pregnant ewes had undetectable or lower ovPAG concentrations than those described in pregnant females (22). PAG profiles in pregnant ewes (23, 83 - 85) are quite different than those obtained in cattle (21). In Churra and Merino ewes, for example, after a first period of high concentrations around Day 60 of gestation, the concentrations decrease until Day 90 and increase again to remain elevated and stable until parturition (23). After parturition, the rate of decline in PAG concentrations is faster in ewes (4 weeks) than in cows (about 14 weeks) (23).

Pregnancy diagnosis in ewes

Pregnancy diagnosis by PAG RIA can be performed in both plasma (86 - 89), and milk samples (90). The measurement of peripheral concentrations of PAG is a reliable method for early pregnancy diagnosis at farm conditions, having equivalent results than progesterone determination and even ultrasound (86, 87, 91). In this aspect, the determination of plasmatic concentrations of PAG/PSPB molecules in ewes can give useful information to develop appropriate feeding strategies for pregnant females and to insure requirements of the mother and fetuses growing in order to avoid metabolic disorders associated to pregnancy (92).

Three different PAG molecules were isolated and partially characterized from goat placenta (15). These proteins differed in amino acid sequence and apparent molecular masses (55, 59 and 62), showing several isoforms with different pI: $caPAG_{55kDa}$ (pI: 5.3, 5.1, 4.9), $caPAG_{59kDa}$ (pI: 6.2, 5.9, 5.6) and $caPAG_{62kDa}$ (pI: 5.1, 4.8). Caprine PAG showed high sequence homology to each other (60 to 73 % residues identical) and a high sequence identity (from 30 to 81 % between the first 27 amino acids sequenced) with proteins of the aspartic proteinase family like boPAG–1, ovPAG–1, boPAG–2, SBU-3 rabbit pepsinogen F and horse PAG (15). In 2000, Garbayo *et al.* described a total of 11 cDNA coding for PAG molecules in caprine placental libraries (caPAG–1 to caPAG–11) (28). Nine of those molecules (caPAG–1, caPAG–3 to –7 and caPAG–9 to –11, named caPAG–1 group) displayed more than 80% sequence identity with each other, expressed in trophoblast binucleate cells. By contrast, caPAG–2 (as boPAG–2 and ovPAG–2) was found to be expressed throughout the trophectoderm during early pregnancy (Days 18 and 19). Also as described in bovine species, caPAG–2 is of more ancient origin than caPAG–1 group, being more recent than caPAG–8.

PAG Radioimmunoassay

The use of two semi-purified preparations (one containing the $caPAG_{55kDa}$ and $caPAG_{59kDa}$ forms, and the other containing the $caPAG_{55kDa}$ and $caPAG_{62kDa}$) allowed the production of two antisera (AS#706 and AS#708) largely used for the development of accurate RIA systems. By use of RIA-706 and RIA-708, PAG concentrations are significantly higher in pregnant goats than in non-pregnant ones as early as 21 days after artificial insemination (24). During gestation, PAG concentrations reach maximal levels in week 8, decrease between weeks 12 and 14 and remain relatively constant until parturition (93, 94). After parturition, concentrations decrease rapidly reaching lower levels at the 4^{th} week postpartum (95). As observed in cattle (65, 96), both number and genotype of fetus can influence PAG concentrations over gestation. From days 21-24 and throughout gestation, twin bearing goats have higher PAG (or PSPB) concentrations than goats bearing one fetus (94, 95, 97). PAG levels in maternal circulation of inter-specific pregnancies (spanish ibex embryos transferred to domestic goats) are also higher (about ten times) when compared to that found in normal intra-specific gestations (98).

Sequential measurement of PAG in goats also allows for the determination of the onset of the disturbance of trophoblastic activity associated with the death of a fetus (99 - 101). As demonstrated by Zarrouk *et al*., in goats carrying a one single fetus, PAG levels fall under the positive pregnancy threshold when the trophoblast died, while in goats carrying 2 or 3 fetuses, analysis of the profiles clearly shows marked drops in concentration that could indicate placental distress at different times (99). Therefore, systematic application of this test in herds with

a high rate of pregnancy failure could help to identify endocrinological phenomena which might be implicated in triggering these events.

Pregnancy diagnosis in caprine species

The use of specific antisera (AS#706 and AS#708) allowed the discrimination between pregnant and non pregnant goats as early as 21 days after breeding (24). However, its use in farm conditions is recommended after Day 26 and 32 in plasma and milk samples, respectively (102, 103).

IDENTIFICATION OF PREGNANCY-ASSOCIATED (SPECIFIC) PROTEINS IN WILD RUMINANTS

Serologically similar antigens to PAG or PSPB have been found in maternal circulation of wild ruminants like mule deer (104), white-tailed deer (104, 105), mountain goats (106), red deer (107), musk-oxen (108), bison (109), moose (110, 111), sika deer (112, 113), elk (111, 114), fallow deer (115 -117) and reindeer (118, 119). The recent isolation and characterization of new forms of pregnancy-associated (specific) proteins from bison (17), buffalo (16), elk and moose placenta (18), as well as the identification of cDNA coding for several PAG molecules (cePAG–1 to –9) in white-tailed deer (120) will allow the development of new investigations concerning plasmatic concentrations, the structure and the phylogenetic origin of this large family of placenta-expressed aspartic proteinase glycoproteins.

CONCLUSION

In conclusion, the family of the Pregnant Associated Glycoproteins appears as a heterologous subgroup of the aspartic proteases. First discovered in ruminants, they are also expressed in other mammals species. Their synthesis in the superficial layers of the trophoblast can be followed by their release in the maternal blood circulation. So, the use of the determination of their concentration can help for pregnancy disgnosis and for new investigations on embryonic or fetal mortalities.

Acknowledgments: This review is part of a study supported by grant from Ministry of the Wallonne Region-DGA (Grant number E'2005-D31-1118) to J.F. Beckers. The authors thank Mrs. R. Fares for her secretarial assistance.

REFERENCES

1. Butler JE, Hamilton WC, Sasser RG, Ruder CA, Hass GM, Williams RJ. Detection and partial characterization of two bovine pregnancy-specific proteins. *Biol Reprod* 1982; 26: 925-933.
2. Lynch KA, Alexander RM, Sasser, RG. The cloning and expression of the bovine pregnancy specific protein B (bPSPB) gene. *Biol Reprod* 1992; 46 (Suppl. 1), 73.

3. Mialon MM, Camous S, Renand G, Martal J, Menissier F. Peripheral concentrations of a 60-kDa pregnancy serum protein during gestation and after calving and in relationship to embryonic mortality in cattle. *Reprod Nutr Dev* 1993; 33: 269-282.
4. Atkinson YH, Gogolin-Ewens KJ, Hounsel EF, Davies MJ, Brandon MR, Seamark RF. Characterization of placentation-specific binucleate cell glycoproteins possessing a novel carbohydrate. *J Biol Chem* 1993; 268: 26679-26685.
5. Beckers JF, Dewulf M, Verstegen J, Wouters-Ballman P, Ectors F. Isolation of a bovine chorionic gonadotrophin (bCG). *Theriogenology* 1988; 29: 218.
6. Beckers JF, Wouters-Ballman P, Ectors F. Isolation and radioimmunoassay of a bovine pregnancy specific protein. *Theriogenology* 1988; 29:219.
7. Zoli AP, Beckers JF, Wouters-Ballman P, Closset J, Falmagne P, Ectors F. Purification and characterization of a bovine pregnancy-associated glycoprotein. *Biol Reprod* 1991; 45: 1-10.
8. Xie S, Low RC, Nagel RJ, et al. Identification of the major pregnancy-specific antigens of cattle and sheep as inactive members of the aspartic proteinase family. *Proc Natl Acad Sci USA* 1991; 88: 10247-10251.
9. Wooding FB. Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. *Placenta* 1992; 13:101-113.
10. Sousa NM, Remy B, El Amiri B, et al. Characterization of pregnancy-associated glycoproteins extracted from zebu (Bos indicus) placentas removed at different gestational periods. *Reprod Nutr Dev* 2002; 42(3): 227-241.
11. Klisch K, De Sousa NM, Beckers JF, Leiser R, Pich A. Pregnancy associated glycoprotein-1, -6, -7, and -17 are major products of bovine binucleate trophoblast giant cells at midpregnancy. *Mol Reprod Dev* 2005; 71(4): 453-460.
12. Xie S, Green JA, Bao B, *et al.* Multiple pregnancy-associated glycoproteins are secreted by day 100 ovine placental tissue. *Biol Reprod* 1997; 57: 1384-1393.
13. El Amiri B, Remy B, Sousa NM, *et al.* Isolation and partial characterization of three pregnancy-associated glycoproteins from the ewe placenta. *Mol Reprod Dev* 2003; 64(2): 199-206.
14. El Amiri B, Remy B, De Sousa NM, et al. Isolation and characterization of eight pregnancy-associated glycoproteins present at high levels in the ovine placenta between day 60 and day 100 of gestation. *Reprod Nutr Dev* 2004; 44(3): 169-181.
15. Garbayo JM, Remy B, Alabart JL, et al. Isolation and partial characterization of a pregnancy-associated glycoprotein family from the goat placenta. *Biol Reprod* 1998; 58: 109-115.
16. Barbato O, Sousa NM, Klisch K, et al. Isolation of pregnancy-associated glycoproteins from water buffalo (Bubalus bubalis) placenta by Vicia villosa affinity chromatography. submitted.
17. Kiewisz J, Sousa NM, Beckers JF, Vervaecke H, Panasiewicz G, Szafranska B. Isolation of pregnancy-associated glycoproteins from placenta of American bison (Bison bison) at first half of pregnancy. *Reprod Fertil Dev* (CSIRO Publishing, Australia) 2006; submitted.
18. Huang F, Cockrell DC, Stephenson TR, Noyes JH, Sasser RG. Isolation, purification, and characterization of pregnancy-specific protein B from elk and moose placenta. *Biol Reprod* 1999; 61:1056-1061.
19. Sasser RG, Ruder CA, Ivani KA, Butler JE, Hamilton WC. Detection of pregnancy by radioimmunoassay of a novel pregnancy-specific protein in serum of cows and a profile of serum concentrations during gestation. *Biol Reprod* 1986; 35: 936-942.
20. Humblot P, Camous S, Martal J, et al. Diagnosis of pregnancy by radioimmunoassay of a pregnancy-specific protein in the plasma of dairy cows. *Theriogenology* 1988; 30: 257-268.
21. Zoli AP, Guilbault LA, Delahaut P, Benitez-Ortiz W, Beckers JF. Radioimmunoassay of a bovine pregnancy-associated glycoprotein in serum: its application in pregnancy diagnosis. *Biol Reprod* 1992; 46: 83-92.

22. El Amiri B, Sulon J, Karen A, et al. Development and validation of two homologous radioimmunoassays for measuring pregnancy-associated glycoprotein (PAG) concentrations in ewe. *Reprod Domest Anim* 2006; in press.
23. Ranilla MJ, Sulon J, Carro MD, Mantecón AR, Beckers JF. Plasmatic profiles of pregnancy-associated glycoprotein and progesterone levels during gestation in churra and merino sheep. *Theriogenology* 1994; 42: 537-545.
24. Gonzalez F, Sulon J, Garbayo JM, et al. Early pregnancy diagnosis in goats by determination of pregnancy-associated glycoprotein concentrations in plasma samples. *Theriogenology* 1999; 52: 717-725.
25. Ayad A, Sousa NM, Sulon J, Iguer-Ouada M, Beckers JF. Comparison of five radioimmunoassay systems for PAG measurement: ability to detect early pregnancy in cows. *Reprod Domest Anim* 2006; in press.
26. Green JA, Parks TE, Avalle MP, et al. The establishment of an ELISA for the detection of pregnancy-associated glycoproteins (PAGs) in the serum of pregnant cows and heifers. *Theriogenology* 2005; 63: 1481-1503.
27. Green JA, Xie S, Quan X, et al. Pregnancy-associated bovine and ovine glycoproteins exhibit spatially and temporally distinct expression patterns during pregnancy. *Biol Reprod* 2000; 62: 1624-1631.
28. Garbayo JM, Green JA, Manikkam M, et al. Caprine pregnancy-associated glycoproteins (PAG): their cloning, expression, and evolutionary relationship to other PAG. *Mol Reprod Dev* 2000; 57(4): 311-322.
29. Hughes AL, Green JA, Garbayo JM, Roberts RM. Adaptive diversification within a large family of recently duplicated, placentally expressed genes. *Proc Natl Acad Sci USA* 2000; 97(7): 3319-3323.
30. Klisch K, Boos A, Friedrich M, et al. The glycosylation of pregnancy-associated glycoproteins and prolactin-related protein-I in bovine binucleate trophoblast giant cells changes before parturition. *Reproduction* 2006; 132: 791-798.
31. Klisch K, Leiser R. In bovine binucleate trophoblast giant cells, pregnancy-associated glycoproteins and placental prolactin-related protein-I are conjugated to asparagine-linked N-acetylgalactosaminyl glycans. *Histochem Cell Biol* 2003; 119: 211-217.
32. Xie S, Low BG, Nagel RJ, Beckers JF, Roberts RM. A novel glycoprotein of the aspartic proteinase gene family expressed in bovine placental trophectoderm. *Biol Reprod* 1994; 51: 1145-1153.
33. Guruprasad K, Blundell TL, Xie S, et al. Comparative modeling and analysis of amino acid substitutions suggests that the family of pregnancy-associated glycoproteins includes both active and inactive aspartic proteinases. *Protein Engineer* 1996; 9: 849-856.
34. Patel OV, Takahashi T, Imai K, Hashizume K. Generation and purification of recombinant bovine pregnancy associated glycoprotein. *Vet J* 2004; 168(3): 328-335.
35. Xie S, Green JA, Bao B, et al. The diversity and evolutionary relationships of the pregnancy-associated glycoproteins, an aspartic proteinase subfamily consisting of many trophoblast-expressed genes. *Proc Natl Acad Sci USA* 1997; 94: 12809-12816.
36. Garbayo JM, Serrano B, Aguilar B, Lopez-Gatius F. Identification of pregnancy-associated glycoproteins (PAGs) expressed by preimplantation embryos of domestic ruminants - direct submission to GenBank (Acc. No.: AY911498 for bPAG22), 2005.
37. Green JA, Xie S, Szafranska B, et al. Identification of a new aspartic proteinase expressed by the outer chorionic cell layer of the equine placenta. *Biol Reprod* 1999; 60: 1069-1077.
38. Gan X, Xie S, Green JA, Roberts RM. Identification of transcripts for pregnancy associated glycoprotein (PAG) in carnivora and perissodactyla. *Biol Reprod* 1997; 56 (Suppl): 431.
39. Kageyama T, Tanabe K, Koiwai O. Structure and development of rabbit pepsinogens. Stage-specific zymogens, nucleotide sequences of cDNAs, molecular evolution, and gene expression during development. *J Biol Chem* 1990; 265(28): 17031-17038.

40. Kageyama T, Ichinose M, Tsukada-Kato S, et al. Molecular cloning of neonate/infant-specific pepsinogens from rat stomach mucosa and their expressional change during development. *Biochem Biophys Res Commun* 2000; 267(3): 806-812.
41. Chen X, Rosenfeld CS, Roberts RM, Green JA. An aspartic proteinase expressed in the yolk sac and neonatal stomach of the mouse. *Biol Reprod* 2001; 65(4): 1092-1101.
42. Hughes AL, Green JA, Piontkivska H, Roberts RM. Aspartic proteinase phylogeny and the origin of pregnancy-associated glycoproteins. *Mol Biol Evol* 2003; 20(11): 1940-1945.
43. Weeth HJ, Herman HA. A histological and histochemical study of the bovine oviducts, uterus and placenta. *Res Bull Univ Miss Agric Exp* 1952; 501.
44. Björkman N. Morphological and histochemical studies on the bovine placenta. *Acta Anat* 1954; 22(Suppl. 2): 1-31.
45. Foote WC, Kaushik DK. LH activity in bovine reproductive tissue. *J Anim Sci* 1963; 22: 857-858.
46. Parlow AF. Bioassay of pituitary luteinizing hormone by depletion of ovarian ascorbic acid. In A. Albert and C. Thomas C (eds), Human pituitary gonadotropins, 1961, pp. 300-310.
47. Ailenberg M, Shemesh M. Partial purification of a chorionic gonadotropin-like protein from bovine cotyledons. *Biol Reprod* 1983; 28: 517-522.
48. Ramachandran J, Sairam MR. The effect of interstitial cell-stimulating hormones, its subunits and recombinants on isolated rat Leydig cell. *Arch Biochem Biophys* 1975; 167: 294-300.
49. Beckers JF, Verstegen J, Dewulf M, Wouters-Ballman P, Ectors F. Purification of bovine chorionic gonadotrophin (bCG). First European Congress of Endocrinology. Copenhagen 21-25 June 1987.
50. Willey KP, Leidenberger F. Functionally distinct agonist and receptor-binding regions in human chorionic gonadotropin. *J Biol Chem* 1989; 33: 19716-19729.
51. Nilson JH, Bokar JA, Clay CM, et al. Different combinations of regulatory elements may explain why placenta-specific expression of the glycoprotein hormone α-subunit gene occurs only in primates and horses. *Biol Reprod* 1991; 44: 231-237.
52. Beckers JF, Roberts RM, Zoli AP, Ectors F, Derivaux J. Molecules of the family of aspartic proteinases in the placenta of ruminants: hormones or proteins ? *Bull Mem Acad R Med Belg* 1994; 149: 355-367.
53. Xie S, Nagel RJ, Green JA, Beckers JF, Roberts RM. Trophoblast-specific processing and phosphorylation of pregnancy-associated glycoprotein-1 in day 15 to 25 sheep placenta. *Biol Reprod* 1996; 54: 122-129.
54. Ushizawa K, Herath CB, Kaneyama K, et al. cDNA microarray analysis of bovine embryo gene expression profiles during the pre-implantation period. *Reprod Biol Endocrinol* 2004; 2: 77.
55. Zoli AP, Demez P, Beckers JF, Reznik M, Beckers A. Light and electron microscopic immunolocalization of bovine pregnancy-associated glycoprotein in the bovine placentome. *Biol Reprod* 1992; 46: 623-629.
56. Zoli AP, Beckers JF, Ectors F. Evidence of a protein immunologically related to pregnant specific proteins in ruminant gonads. 3th International Ruminant Reproduction Symposium (Nice) 1990; 304.
57. Zoli AP, Ectors F, Beckers JF. Ruminant gonads as accessory sources of pregnant specific protein ? 72nd Annual Meeting of the Endocrine Society (Atlanta) 1990; 118.
58. Green JA, Xie S, Roberts RM. Pepsin-related molecules excreted by trophoblast. *Rev Reprod* 1998; 3: 62-69.
59. Wooding FB, Roberts RM, Green JA. Light and electron microscope immunocytochemical studies of the distribution of pregnancy associated glycoproteins (PAGs) throughout pregnancy in the cow: possible functional implications. *Placenta* 2005; 26: 807-827.

60. Ushizawa K, Takahashi T, Kaneyama K, Tokunaga T, Tsunoda Y, Hashizume K. Gene expression profiles of bovine trophoblastic cell line (BT-1) analyzed by a custom cDNA microarray. *J Reprod Dev* 2005; 51: 211-220.
61. Ishiwata H, Katsuma S, Kizaki K, et al. Characterization of gene expression profiles in early bovine pregnancy using a custom cDNA microarray. *Mol Reprod Dev* 2003; 65: 9-18.
62. Kirakofe GH, Wright JM, Schalles RR, Ruder CA, Paris S, Sasser RG. Pregnancy-specific protein B in serum of postpartum beef cows. *J Anim Sci* 1993; 71: 2199-2205.
63. Ali M, Gebbie FE, Sinclair KD, et al. Half-life of serum bovine pregnancy-associated glycoprotin (bPAG), postpartum anoestrus and performance of beef cows. *J Reprod Fert* 1997; Abstract Series 20, 25.
64. Haugejorden G, Waage S, Dahl E, Karlberg K, Beckers JF, Ropstad E. Pregnancy associated glycoproteins (PAG) in postpartum cows, ewes, goats and their offspring. *Theriogenology* 2006; 66: 1976-1984.
65. Guilbault LA, Beckers JF, Lapierre S, Zoli AP, Benitez-Ortiz W, Roy GL. Peripartum concentrations of placental protein hormones (hPL and bPAG) in holstein and hereford recipients carrying pubered holstein fetuses. *Theriogenology* 1991; 35: 208.
66. Kornmatitsuk B, Veronesi MC, Madej A, et al. Hormonal measurements in late pregnancy and parturition in dairy cows-possible tools to monitor fetal well being. *Anim Reprod Sci* 2002; 72(3-4): 153-164.
67. Perényi Z, Szenci O, Drion PV, et al. Aspartic proteinase members secreted by the ruminant placenta: specificity of three radioimmunoassay systems for the measurement of pregnancy-associated glycoproteins. *Reprod Domest Anim* 2002; 37:324-329.
68. Perényi Z, Szenci O, Sulon J, Drion PV, Beckers JF. Comparison of the ability of three radioimmunoassays to detect pregnancy-associated glycoproteins in bovine plasma. *Reprod Domest Anim* 2002; 37: 100-104.
69. Chavatte-Palmer P, de Sousa N, Laigre P, et al. Ultrasound fetal measurements and pregnancy associated glycoprotein secretion in early pregnancy in cattle recipients carrying somatic clones. *Theriogenology* 2006; 66: 1004-1011.
70. Lopez-Gatius F, Garbayo JM, Santolaria P, et al. Milk production correlates negatively with plasma levels of pregnancy-associated glycoprotein (PAG) during the early fetal period in high producing dairy cows with live fetuses. *Domest Anim Endocrinol* 2007; 32: 29-42.
71. Humblot P, Camous S, Martal J, et al. Pregnancy specific protein B, progesterone concentrations and embryonic mortality during early pregnancy in dairy cows. *J Reprod Fertil* 1988; 83: 215-223.
72. Szenci O, Humblot P, Beckers JF, et al. Plasma profiles of progesterone and conceptus proteins in cows with spontaneous embryonic/foetal mortality as diagnosed by ultrasonography. *Vet J* 1999; 159: 287-290.
73. Szenci O, Taverne MA, Beckers JF, et al. Evaluation of false ultrasonographic diagnoses in cows by measuring plasma levels of bovine pregnancy-associated glycoprotein 1. *Vet Rec* 1998; 142: 304-306.
74. Szenci O, Beckers JF, Humblot P, et al. Comparison of ultrasonography, bovine pregnancy-specific protein B, and bovine pregnancy-associated glycoprotein 1 tests for pregnancy detection in dairy cows. *Theriogenology* 1998; 50: 77-88.
75. Taverne MA, Breukelman SP, Perenyi Z, et al. The monitoring of bovine pregnancies derived from transfer of in vitro produced embryos. *Reprod Nutr Dev* 2002; 42(6): 613-624.
76. Breukelman SP, Perenyi Z, Ruigh L, et al. Plasma concentrations of bovine pregnancy-associated glycoprotein (bPAG) do not differ during the first 119 days between ongoing pregnancies derived by transfer of in vivo and in vitro produced embryos. *Theriogenology* 2005; 63(5): 1378-1389.

77. Semambo DKN, Eckersall PD, Sasser RG, Ayliffe TR. Pregnancy-specific protein B and progesterone in monitoring viability of the embryo in early pregnancy in the cow after experimental infection with Actynomyces pyogenes. *Theriogenology* 1992; 37: 741-748.
78. Ruder CA, Stellflug JN, Dahmen JJ, Sasser RG Detection of pregnancy in sheep by radioimmunoassay of sera for pregnancy-specific protein B. *Theriogenology* 1988; 29: 905-912.
79. Zoli AP, Beckers JF, Ectors F. Isolement et caractérisation partielle d'une glycoprotéine associée à la gestation chez la brebis. *Ann Méd Vét* 1995; 139 :177-184.
80. Willard JM, White DR, Wesson CAR, Stellflug J, Sasser RG. Detection of fetal twins in sheep using a radioimmunoassay for pregnancy-specific protein B. *J Anim Sci* 1995; 73: 960-966.
81. Nagel RJ, Xie S, Roberts RM. Aspartic proteinases as markers of trophoblast differentiation in sheep. *Biol Reprod* 1993; 48 (Suppl.): 139.
82. Gogolin-Ewens KJ, Lee CS, Mercer WR, Moseby AM, Brandon MR. Characterization of a sheep trophoblast-derived antigen first appearing at implantation. *Placenta* 1986; 7: 243-255.
83. Ranilla MJ, Sulon J, Mantecón AR, Beckers JF, Carro MD. Plasma pregnancy-associated glycoprotein and progesterone concentrations in pregnant assaf ewes carrying single and twin lambs. *Small Rum Res* 1997; 24: 125-131.
84. Gajewski Z, Beckers JF, Sousa NM, Thun R, Sulon J, Faundez R. Oznaczanie stezenia PAG u owiec badania retrospektywne. *Adv Cell Biol* 1999; 26 (Suppl.12): 89-96.
85. Ledezma-Torres RA, Beckers JF, Holtz W. Assessment of plasma profile of pregnancy-associated glycoprotein (PAG) in sheep with a heterologous (anti-caPAG(55+59)) RIA and its potential for diagnosing pregnancy. *Theriogenology* 2006; 66: 906-912.
86. Karen A, Beckers JF, Sulon J, et al. Evaluation of false transrectal ultrasonographic pregnancy diagnoses in sheep by measuring the plasma level of pregnancy-associated glycoproteins. *Reprod Nutr Dev* 2003; 43: 577-586.
87. Karen A, Beckers JF, Sulon J, et al. Early pregnancy diagnosis in sheep by progesterone and pregnancy-associated glycoprotein tests. *Theriogenology* 2003; 59: 1941-1948.
88. Verberckmoes S, Vandaele L, De Cat S, et al. A new test for early pregnancy diagnosis in sheep: determination of ovine pregnancy-associated glycoprotein (ovPAG) concentration by means of a homologous radioimmunoassay. *Flemish Vet J* 2004; 73: 119-127.
89. Vandaele L, Verberckmoes S, El Amiri B, et al. Use of a homologous radioimmunoassay (RIA) system to evaluate the effect of maternal and foetal parameters on pregnancy-associated glycoprotein (PAG) concentration in sheep. *Theriogenology* 2005; 63: 1914-1924.
90. El Amiri B, Cognié Y, Sulon J, et al. Pregnancy-associated glycoprotein concentrations in plasma and milk samples for early pregnancy diagnosis in Lacaune dairy sheep. *Reprod Domest Anim* 2003; 38(4): 322.
91. Karen A, Amiri BE, Beckers JF, Sulon J, Taverne MA, Szenci O. Comparison of accuracy of transabdominal ultrasonography, progesterone and pregnancy-associated glycoproteins tests for discrimination between single and multiple pregnancy in sheep. *Theriogenology* 2006; 66(2): 314-322.
92. El Amiri B, Karen A, Cognié Y, et al. Diagnostic et suivi de gestation chez la brebis: réalités et perspectives. *Prod Anim INRA* 2003; 16 : 79-90.
93. Folch J, Benitez-Ortiz W, Alabart JL, Beckers JF. Determinacion de la concentracion plasmatica de PAG (pregnancy-associated glycoprotein) en cabras blanca celtibčrica y su utilizacion como diagnostico de gestacion. *ITEA* 1993; 12: 364-366.
94. González F, Sulon J, Garbayo JM, et al. Secretory profiles of pregnancy-associated glycoproteins at different stages of pregnancy in the goat. *Reprod Domest Anim* 2000; 35: 65-68.
95. Sousa NM, Garbayo JM, Figueiredo JR, Sulon J, Gonçalves PBD, Beckers JF. Pregnancy-associated glycoprotein and progesterone profiles during pregnancy and postpartum in native goats from the north-east of Brazil. *Small Rum Res* 1999; 32: 137-147.

96. Patel OV, Sulon J, Beckers JF, et al. Plasma bovine pregnancy-associated glycoprotein concentrations throughout gestation in relationship to fetal number in the cow. *Eur J Endocrinol* 1997; 137: 423-428.
97. Humblot P, Montigny G, Jeanguyot N, et al. Pregnancy-specific protein B and progesterone concentrations in french alpine goats throughout gestation. *J Reprod Fert* 1990; 89: 205-212.
98. Fernández-Arias A, Alabart JL, Folch J, Beckers JF. Interspecies pregnancy of Spanish ibex (Capra pyrenaica) fetus in domestic goat (Capra hircus) recipients induces abnormally high plasmatic levels of pregnancy-associated glycoprotein. *Theriogenology* 1999; 51: 1419-1430.
99. Zarrouk A, Engeland I, Sulon J, Beckers JF. Determination of pregnancy-associated glycoprotein concentrations in goats (Capra hircus) with unsuccessful pregnancies: a retrospective study. *Theriogenology* 1999; 51: 1221-1231.
100. Zarrouk A, Engeland IV, Sulon J, Beckers JF. Pregnancy-associated glycoprotein levels in pregnant goats inoculated with Toxoplasma gondii or Listeria monocytogenes: a retrospective study. *Theriogenology* 1999; 52:1095-1104.
101. Faye D, Sulon J, Kane Y, et al. Effects of an experimental Trypanosoma congolense infection on the reproductive performance of West African Dwarf goats. *Theriogenology* 2004; 62(8): 1438-1451.
102. Gonzalez F, Sulon J, Calero P, Batista M, Gracia A, Beckers JF. Pregnancy-associated glycoproteins (PAG) detection in milk samples for pregnancy diagnosis in dairy goats. *Theriogenology* 2001; 56(4): 671-676.
103. Gonzalez F, Cabrera F, Batista M, et al. A comparison of diagnosis of pregnancy in the goat via transrectal ultrasound scanning, progesterone, and pregnancy-associated glycoprotein assays. *Theriogenology* 2004; 62(6): 1108-1115.
104. Wood AK, Short RE, Darling AE, Dusek GL, Sasser RG, Ruder CA. Serum assays for detecting pregnancy in mule and white-tailed deer. *J Wild Manag* 1986; 50: 684-687.
105. Osborn DA, Beckers JF, Sulon J, et al. Use of glycoprotein assays for pregnancy diagnosis in white-tailed deer. *J Wild Manage* 1996; 60: 388-393.
106. Houston BD, Robbins CT, Ruder CA, Sasser RG. Pregnancy detection in mountain goats by assay for PSPB. *J Wild Manage* 1986; 50: 740-742.
107. Haigh JC, Cranfield M, Sasser RG. Estrus synchronization and pregnancy diagnosis in red deer. *J Zoo Anim Med* 1988; 19:202-207.
108. Rowell JE, Flood PF, Ruder CA, Sasser RG. Pregnancy-associated protein in the plasma of captive musk-oxen. *J Wild Manag* 1989; 53: 899-901.
109. Haigh JC, Gates C, Ruder A, Sasser RG. Diagnosis of pregnancy in wood bison using a bovine assay for pregnancy-specific protein B. *Theriogenology* 1991; 36:749-754.
110. Haigh JC, Dalton WJ, Ruder CA, Sasser RG. Diagnosis of pregnancy in moose using a bovine assay for PSPB. *Theriogenology* 1993; 40:905-911.
111. Huang F, Cockrell DC, Stephenson TR, Noyes JH, Sasser RG. A serum pregnancy test with a specific radioimmunoassay for moose and elk pregnancy-specific protein *B*. *J Wild Manag* 2000; 64: 492-499.
112. Willard ST, Sasser RG, Bringans M, et al. Methods for pregnancy detection in sika deer (Cervus nippon). *J Anim Sci* 1994; 72(Suppl. 1): 1309.
113. Willard ST, Hughes DMJr, Bringans M, et al. Artificial insemination, hybridization and pregnancy detection in sika deer (Cervus nippon). *Theriogenology* 1996; 46:779-789.
114. Willard ST, Sasser RG, Gillespie JC, et al. Methods for pregnancy determination and the effects of body condition on pregnancy status in rocky mountain elk (Cervus elephus nelsoni). *Theriogenology* 1994; 42:1095-1102.

115. Willard ST, Sasser RG, Gillespie JC, Jacques JT, Welsh TH, Randel RD. Early pregnancy detection and embryonic mortality in fallow deer (Dama dama). *J Anim Sci* 1994; 72(Suppl. 2), 67.
116. Willard ST, Sasser RG, Jaques JT, White DR, Neuendorff DA, Randel RD. Early pregnancy detection and the hormonal characterization of embryonic-fetal mortality in fallow deer (Dama dama). *Theriogenology* 1998; 49: 861-869.
117. Willard ST, Petty SJ, Sasser RG, White DR, Randel RD. Pregnancy detection and the effects of age, body weight, and previous reproductive performance on pregnancy status and weaning rates of farmed fallow deer (Dama dama). *J Anim Sci* 1999; 77: 32-38.
118. Russel DE, Gerhart KL, White RG, Wetering DVD. Detection of early pregnancy in caribou: evidence for embryonic mortality. *J Wild Manag* 1998; 62: 1066-1075.
119. Ropstad E, Johansen O, King C, et al. Comparison of plasma progesterone, transrectal ultrasound and pregnancy specific proteins (PSPB) used for pregnancy diagnosis in reindeer. *Acta Vet Scand* 1999; 40: 151-162.
120. Vawter GA, Parks TE, Green JA, Ealy AD, Killian G. Pregnancy-associated glycoproteins from Odocoileus virginianus (white-tailed deer) placentas. Direct submission to GenBank (2003).

Received: September 15, 2006
Accepted: October 16, 2006

Author's address: Professor J.F. Beckers, Ph.D., DVM, Physiology of Animal Reproduction, Faculty of Veterinary Medicine, University of Liege, B-4000, Belgium. Phone: +32-43664161, Fax: +32-43664165; e-mail: jfbeckers@ulg.ac.be