THE PREGNANCY-ASSOCIATED GLYCOPROTEINS: BIOCHEMICAL ASPECTS AND CLINICAL APPLICATION FOR PREGNANCY FOLLOW-UP

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SUMMARY

Pregnancy diagnosis is an important part in reproduction management of ruminants. In the last years, a large polymorphic family of placenta-expressed proteins has been discovered in ruminant species and used for pregnancy diagnosis. Members of this family are named pregnancy-associated glycoproteins (PAG), being synthesized in the mono- and binucleate cells of the ruminant's trophectoderm. Part of them are released in the maternal blood circulation where they can be assayed by different laboratory techniques. Due to large variety of expressed molecules and to large variations in the post-translational processing of the PAG, different immuno-systems present different ability to quantify the PAG released in blood. The sensitivity (92 to 100%) and specificity of PAG radioimmunoassay when used for pregnancy diagnosis are very high. The assay of PAG can also bring very interesting information for researchers working in programs focused on the study of embryonic and fetal mortalities, as well as on embryo biotechnology (ET, FIV, clonage), animal nutrition, or infections diseases resulting in pathologies affecting the pregnancy.

INTRODUCTION

Placenta is a polyvalent organic system with a vital biological role for the perpetuation of the eutherian mammals. Fetal development is related to that of the placenta from the anatomical, genetic and metabolic points of view. As far as metabolism is concerned, endocrine function is particularly important. The endocrine function of the mammalian placenta includes various hormones (progesterone, estrogens, chorionic gonadotropins, placental lactogens, growth hormones…), and a series of growth factors and glycoproteins interfering with the establishment of pregnancy, corpus luteum maintenance, immunotolerance of the conceptus by the mother, intermediate maternal metabolism, fetal growth and mammary growth.

The study of the pregnancy-associated glycoproteins in ruminant species will be discussed below. Emphasis will be given to radioimmunoassay methods used for pregnancy diagnosis and for follow-up of placenta secretory function in different physiological or pathological conditions.

1. Binucleate cells of ruminant placenta

Special cells of the ruminant placenta were suspected as responsible for an important endocrine function during gestation as early as 1940. In this decade, it was observed for the fist

time a reduction of the pituitary gonadotropic activity in the pregnant cow and suggested that the corpus luteum was replaced by additional luteotropic substances produced elsewhere. Using microsections of cotyledons and staining for carbohydrates like periodic acid-Schiff (PAS), Weeth and Herman (1952) and Björkman (1954) described the presence of numerous trophoblastic cells containing glycoproteins. About 10 years later, Foote and Kaushik (1963) demonstrated the presence of a LH-like activity in the cotyledons. They used the bioassay of Parlow (1961) in order to identify this LH-like activity. These studies were pioneers in this field: they opened the way for further identification and characterization of placental hormones and proteins in the ruminant species.

The most characteristic element of the ruminant placenta is the presence of binucleate cells or diplokaryocytes in the trophectoderm (Wimsatt, 1951). Binucleate cells derive from chorionic mononucleate cells by karyokinesis without subsequent cytokinesis. The youngest cells are located deep in the trophectoderm and maturation takes place progressively as they rise to the maternal surface (Wooding, 1983). The first binucleate cells appear just before implantation (Wango et al., 1990a). They can be recognized from day 14 after fertilization in the ovine trophectoderm (Wooding, 1984) and from day 16-17 in the bovine.

The binucleate cells represent 15-20% of the total population within the mature placenta (Wooding, 1983; Wango et al., 1990b). Of these, about one in seven is continuously migrating towards the uterine epithelium at any time of gestation (Wooding et al., 1986). The migration of binucleate cells plays an important role in the remodeling of placental structure and leads to a reaction with the uterine cells: the formation of a hybrid feto-maternal tissue in the ewe and goat (the syncytium plaques) and the maternal giant cells in the cow (the trinucleate cells) (Wooding, 1984; Wooding and Beckers, 1987).

Binucleate cells are directly involved in the production of progesterone, prostaglandins, placental lactogen hormones and pregnancy-associated (-specific) glycoproteins (reviewed by Sousa et al., 2001). These secretory products are stored in dense granules which occupy more than 50% of the cytoplasm (Lee et al., 1986) and deliver their content directly in the maternal system after binucleate cell migration (Wooding, 1984).

2. The pregnancy-associated glycoproteins (PAGs) in ruminant species

Characterized in the last 25 years (Butler et al., 1982; Beckers et al., 1988a,b; Zoli et al., 1991; Xie et al., 1991), 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 (Zoli et al., 1992a; Wooding, 1992).

By using biochemical procedures, some molecules of the PAG family were isolated from cotyledons of cow (Zoli et al., 1991; Sousa et al., 2002; Klisch et al., 2005), ewe (Xie et al., 1997a; El Amiri et al., 2003, 2004), goat (Garbayo et al., 1998), buffalo (Barbato et al., 2006), bison (Kiewisz et al., 2007), moose and elk (Huang et al., 1999). Purified and semi-purified preparations were used to immunize rabbits and the antisera (AS) obtained allowed the development of homologous (Sasser et al., 1986; Humblot et al., 1988; Zoli et al., 1992b; Mialon et al., 1993) and heterologous radioimmunoassay (RIA) (Ranilla et al., 1994; González et al., 1999; El Amiri et al., 2006; Ayad et al., 2007), and sandwich enzyme-linked immunosorbant assay (ELISA) systems (Green et al., 2005).

Screening of placental libraries with nucleic acid probes has identified additional (probably more than 100) cDNA that code for PAG molecules (Xie et al., 1997b; Green et al., 2000; Garbayo et al., 2000). Recent investigations have also demonstrated that different PAG cDNA are not expressed coordinately throughout pregnancy (Green et al., 2000; Hughes et al., 2000). Some, for example, are expressed early, others only when pregnancy progresses.

An important general feature of ruminant PAG is that they are extensively glycosylated proteins undergoing a complex post-translational processing, with the carbohydrate signature of the trophoblastic cells (Klisch et al., 2006). 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) (Klisch and Leiser, 2003; Klisch et al., 2005). 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 (Klisch et al., 2005).

3. The major bovine PAG family: the boPAG-1 group

The first PAG purified from bovine fetal cotyledons was named boPAG-1 or boPAG$_{67kDa}$ (Zoli et al., 1991). The boPAG-1, also known under a variety of other names including pregnancy-specific protein B (Butler et al., 1982; Lynch et al., 1992) and pregnancy-specific protein 60 (Mialon et al., 1993), was first described as a placental antigen of cattle placenta which was also present in the blood serum of the mother soon after implantation.

The boPAG-1 purified by Zoli et al. (1991), was shown to be an acidic glycoprotein with 67 kDa molecular mass. Four isoforms (I, II, II and IV) with different isoeletric points (4.4, 4.6, 5.2 and 5.4) were detected in this initial preparation. Each pI was closely related to the amount of sialic acid of the PAG isoform (2.12%; 0.83%, 0.64% and 0.29%, respectively). Moreover, pI, sialic acid content and immunoreactivity were closely related; the most basic form being the most immunoreactive (Zoli et al., 1991).

The most surprising characteristic of boPAG-1, revealed by Roberts and coworkers soon after its purification (Xie et al., 1991), was the fact that this protein belongs to a family of proteolytic enzymes known as aspartic proteinases, with more than 50% amino acid sequence identity to pepsin, cathepsin D and cathepsin E. The boPAG-1 has the bilobed structure typical of all known eukaryotic aspartic proteinases and possesses a cleft between the two lobes capable of accommodating peptides up to 7 amino acid long (Xie et al., 1997b). However, despite its structural similarity with other active aspartic proteinases, this molecule is considered as catalytically inactive because of key mutations close to the active site (Xie et al., 1991).

More 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 (Klisch and Leiser, 2003). By the use of this technique, Klisch et al. (2005) isolated 3 distinct PAG molecules from bovine cotyledons: boPAG$_{56kDa}$, boPAG$_{67kDa}$ and boPAG$_{75kDa}$. 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.

Interestingly, the presence of antigens immunologically similar to boPAG$_{67kDa}$ has also been demonstrated in testicular tissue and in ovarian extracts (Zoli et al., 1990a,b), justifying the

adjective “associated” and not “specific” given to this glycoprotein. 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 (Green et al., 1998; Wooding et al., 2005). However, their high level of expression in early gestation (Ushizawa et al., 2004, 2005) point to a fundamental role of such as molecules in implantation and placentogenesis (Ishiwata et al., 2003).

4. Pregnancy-associated glycoprotein detection by radioimmunoassay

4.1. The classical PAG-497 radioimmunoassay

In 1992, Zoli et al. described the validation of a homologous PAG RIA with the use of bo-PAG_{67kDa} as standard, tracer and immunogen for antiserum production (AS#497). 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 1,000 to 5,000 ng/ml only few days before delivery (Zoli et al., 1992b). Concentrations decrease steadily in the postpartum period reaching undetectable levels only by day 100 postpartum (Zoli et al., 1992b). 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 (Kiracofe et al., 1993; Haugejorden et al., 2006).

4.2. Alternative PAG radioimmunoassays

Interest in improving existing immunoassay methods for PAG by testing new antisera (Perényi et al., 2002a,b; Ayad et al., 2007) can be explained by different phenomena such as the temporal expression of different PAG molecules during early pregnancy and the higher N-terminal amino acid identities but distinct glycosylation patterns (and probably half-life) of PAG molecules purified from bovine placenta. As well, several authors reported a distinct ability of different antisera to detect PAG epitopes during early pregnancy in cattle (Perényi et al., 2002b; Chavatte-Palmer et al., 2006; Lopez-Gatius et al., 2007; Ayad et al., 2007).

Recently, Perényi et al. (2002b) 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). 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 earlier expressed PAG molecules can be better recognized by antisera raised against PAG coming from other species. In the same way, in a preliminary study, we demonstrate that the use of a 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 (Ayad et al., 2007).

4.3. 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 phosphatase-conjuged anti-rabbit antibody. The concentrations of PAG measured by ELISA rose rapidly between Days 24 and 28 of pregnancy (Green et al., 2000). However, the ability of this assay to discriminate open and pregnant females in early gestation was not yet analyzed.

5. Influence of different factors on PAG profiles

PAG concentrations have been directly correlated to the placental mass (Vasques et al., 1995; Patel et al., 1997; Wallace et al., 1997), which in turn is related to the stage of pregnancy (Wooding, 1993; Ferrel, 1991). Investigations realized in peripartum clearly demonstrated a positive influence of both maternal environment and fetal genotype (sex and race) on peripheral blood concentrations of PAG molecules (Zoli et al., 1992b; Guilbault et al., 1991; Kornmatitsuk et al., 2002). Higher PAG concentrations are observed in maternal than in fetal serum, suggesting that this glycoprotein is delivered preferentially in the maternal system (Zoli et al., 1992b).

Another original approach was recently developed by Lopez-Gatius et al. (2007), who determined plasma levels of bovine PAG (measured by both RIA-497 and RIA-706) and progesterone in Holstein Friesian dairy cows. 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. PAG concentrations 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.

6. Pregnancy diagnosis

In practice, the measurement of PAG concentrations in maternal blood allows to confirm pregnancy (the pregnancy diagnosis) as well as to follow the trophoblastic function in research and veterinary practice.

In cattle, concentrations of PAG can be measured from Day 28 (Szenci et al., 1998a,b) to 30 (Sasser et al., 1986; Humblot et al., 1988) after breeding (or IA) with a sensitivity of 92%. Sensitivity increase at Days 37-38 to reach 99-100% (Table 1). The specificity calculated for different works ranged from 80 and 90% mostly due to naturally occurring embryonic mortality.

Table 1. Diagnosis of pregnancy in cattle by the measurement of pregnancy-associated glycoprotein.

Day of gestation	n	Se (%)	Sp (%)	PPV (%)	NPV (%)	References
29-30	138	92.0	82.6	81.6	92.5	Szenci et al., 1998b
35	430	98.9	87.5	93.0	97.9	Zoli et al., 1992b
37-38	125	100	84.0	83.5	100	Szenci et al., 1998b

Se: sensitivity; Sp: specificity; PPV: positive predictive value; NPV: negative predictive value.

In the field, the measurement of PAG was developed for routine analysis in veterinarian laboratories. The leader country is France with almost 100,000 pregnancy diagnoses per year, followed by Belgium (6,000) and other European countries such as Spain and Scotland. Presently, PAG measurements are also starting at the University of Perugia (Italy), with our colleague Dr. Olimpia Barbato.

7. Clinical application of PAG RIA for pregnancy follow-up

The assay of PAG can also bring very interesting information for researchers working in programs focused on embryonic and fetal mortalities, on embryo biotechnology (ET, FIV, somatic nuclear transfer), on animal nutrition, and in infections diseases. Some aspects of PAG concentrations at different pathological conditions will be described below.

7.1. Study of embryonic/fetal mortalities

Figure 1 illustrates results from Szenci et al. (1998a) who followed several cows by ultrasonography, as well as by progesterone and PAG radioimmunoassay. In their study, these authors described different situations in which PAG concentrations allowed to confirm or to elucidate embryonic or fetal mortalities.

A) In the first cow (n. 3129), embryonic mortality occurred before Day 50. At Day 56, the progesterone remained high while the PAG concentrations fell under the threshold, meaning the dead of fetus and placenta.
B) In the second cow (n. 3570), the concentrations of PAG and progesterone clearly showed that until Day 30 the cow was pregnant. At Day 38, progesterone concentrations fell together with those of PAG. The ultrasound performed at Day 38 was also negative.
C) The third cow (n. 3369) was inseminated 2 times at 21 days interval. At Day 40 and 45 after the first insemination, the cow was pregnant. Ultrasound gave a positive result and progesterone concentrations were high. PAG concentration was increasing indicating a normal ongoing pregnancy. However, at Day 54, the cow was not pregnant as indicated by a negative ultrasound and by decreasing PAG and progesterone concentrations. The second AI was probably responsible of the embryonic death occurring 45 days after the first AI.
D) The fourth cow (n. 3004) was inseminated close after parturition. PAG was still high at the time of insemination. After the PAG concentration decreased, progesterone concentrations and ultrasound indicated a pregnancy status at Day 28. At Day 34, the cow was not pregnant as indicated by undetectable PAG and progesterone concentrations as well as by ultrasound examination.

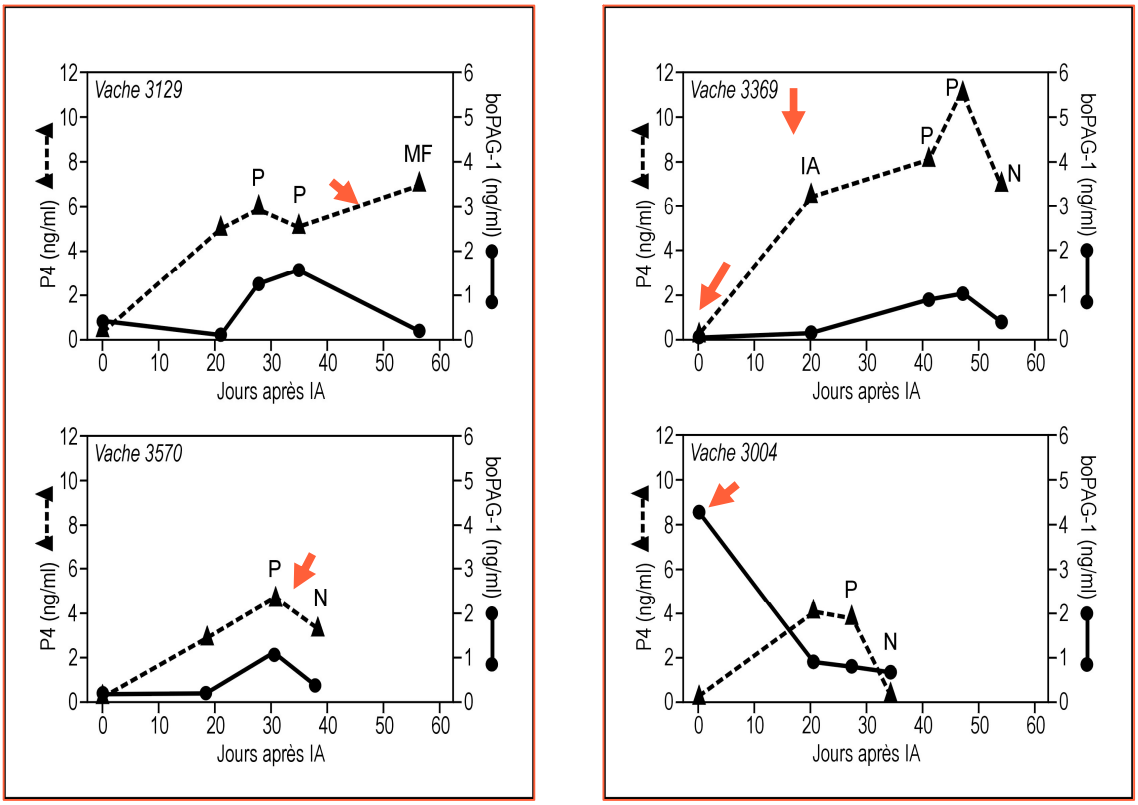

Figure 1. Plasma PAG and progesterone concentrations in four cows in which an initial positive ultrasonographic pregnancy diagnosis (P) was followed by a negative pregnancy diagnosis (N) based on PAG/progesterone concentrations or by direct ultrasonographic observation of fetal death. Day 0 corresponds to day of the first artificial insemination. Arrows indicate the probable time of embryo or fetus death. Dotted line represents the concentration of progesterone. Black and continuous line represents the PAG concentrations. MF means fetal death. (Adapted from Szenci et al., 1998b).

7.2. Early secretion of PAG in unfavorable uterine environment:

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 revealed an atypical case (Figure 2).

The recipient R599 was transferred without an active corpus luteum as revealed by the low progesterone 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 progesterone and the low concentrations of embryotrophic 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.

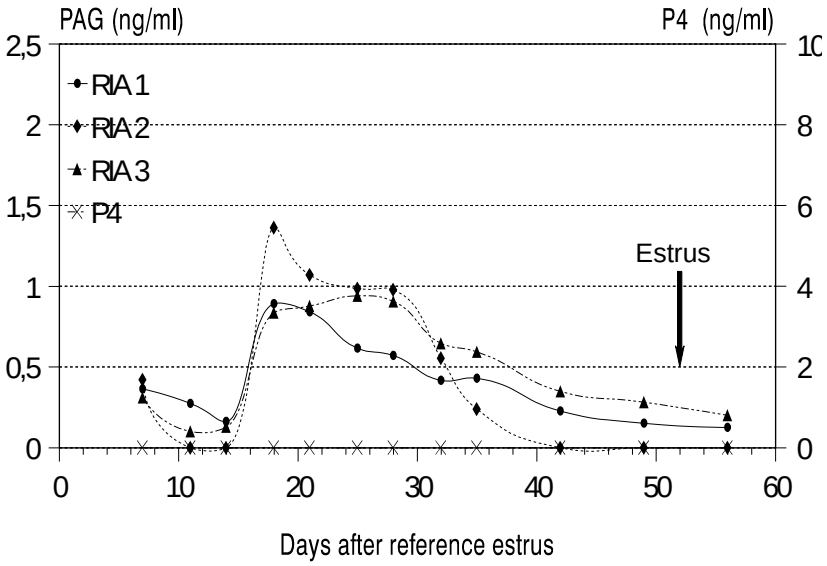

Figure 2. During the observation period the cow n. R599 did not express detectable levels of progesterone, retrospectively, the concentrations of progesterone indicate the lack of an active corpus luteum. This pathology, often observed in north countries in winter, was described as "Functional anoestrus". 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 and 2 (RIA-497 and RIA-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 (adapted from Perenyi, 2002).

7.3. Follow-up of placental secretory function after the use of embryo biotechnology

In nuclear transfer programs, even if the majority of calves are of normal size, some of them can present morphological abnormalities like edema of the umbilical cord with placental hypertrophy. As early as in 1996, Ectors et al. described higher concentrations of PAG in maternal blood of recipient heifers carrying cloned embryos/foetus. In the same way, abnormally high PAG concentrations in maternal circulation were indicative for the presence of a abnormal amount of trophoblast tissues, as observed in hydatiform molar pregnancy (Ectors et al., 1996).

More recently, by analyzing serial PAG determinations (RIA-497, RIA-706 and RIA-708) and successive ultrasound fetal measurements, Chavatte-Palmer et al. (2006) demonstrated that cattle recipients carrying somatic clones showed alterations in development speed. 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 alteration of placentas issued from nuclear transfer techniques.

7.4. Increased PAG concentrations in undernourished cows

PAG concentrations can be modified by environmental conditions such as nutritional status. In a recent study, we followed 12 Azawak zebu cows from Burkina Faso (Sousa et al., 2003). Blood sampling were performed from the 8th week of pregnancy until the 10th week postpartum. Eleven cows showed a very homogeneous profile similar to those described in *Bos taurus* breeds. However, one cow presented a decrease in body condition score associated with very high concentrations of PAG. This finding indicates that the PAG concentration can reflect different events and environmental conditions.

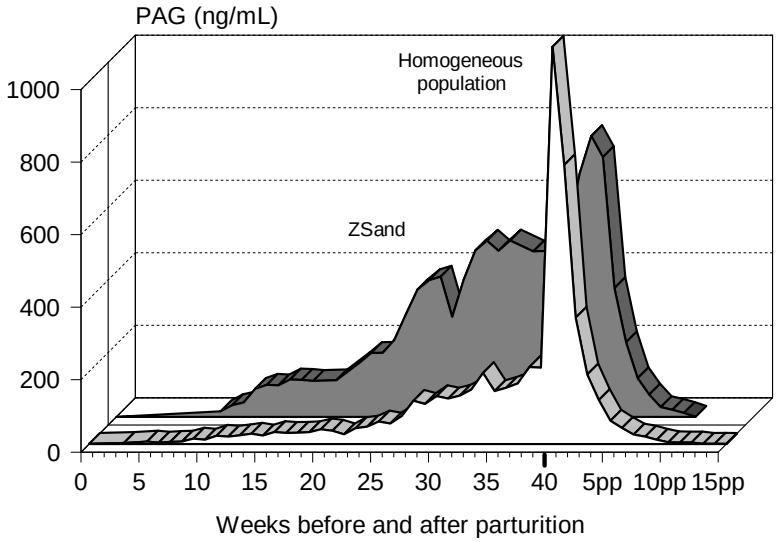

Figure 3. PAG profile of Azawak zebu cows presenting clinically normal gestations (homogeneous population; white color) and PAG profile of the individual cow having presented an extremely low BCS at the end of gestation (Zsand; gray color). Week 40 of gestation corresponds to the Week 0 postpartum (0 pp). (Adapted from Sousa et al., 2003).

CONCLUSION

Thanks to international collaborative studies, we have shown that PAG levels are a good indicator of feto-placental well-being and that sharp decreases in PAG levels occur just before pregnancy failure in cows as well as in other ruminant species. In some countries, the PAG assay is available for Doctors in Veterinary Medicine in the regional laboratories responsible for animal health including immunodiagnosis for brucellosis, IBR, BVD, CAEV, VISNA-MEDI etc.

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