

Original article

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CURRENT TRENDS IN FOLLOW-UP OF TROPHOBLASTIC FUNCTION IN RUMINANT SPECIES

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During the pregnancy of ruminants, different hormones and proteins are secreted by placenta or corpus luteum allowing the follow up of gestation. Among them, progesterone (P4) and pregnancy-associated glycoproteins (PAG) were proposed as laboratory tools to establish or to confirm pregnancy diagnosis. In last years, PAG assay also provided useful information for researchers working in programs focused on the follow up of trophoblastic function. Concentrations of PAG appeared as altered after the use of embryo biotechnology (in vitro fertilization, cloning by nuclear transfer, inter-specific pregnancies), according to nutritional status of pregnant females (overnourished or undernourished), or consecutive to infectious diseases leading to pathologies affecting the pregnancy in cows (*Actinomyces pyogenes* and *Neospora caninum*) and goats (*Toxoplasma gondii*, *Listeria monocytogenes* and *Trypanosoma congolense*). As well, in numerous studies, the association of repeated ultrasound examinations with P4 and PAG determinations allowed a better understanding of mechanisms related to embryonic and fetal mortalities: failure after artificial insemination or embryo transfer techniques, large offspring syndrome after *in vitro* fecundation and cloning.

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

INTRODUCTION

Placenta is a highly specialized organic system. As far as metabolism is concerned, endocrine function is particularly important. The endocrine function of the ruminant placenta includes the synthesis and secretion of various hormones (progesterone (P4), oestrogens, placental lactogens, prolactins),

growth factors, enzymes, inhibitors, among others. Since early eighties, a large family of placental proteins without known biological activities (pregnancy-associated or -specific proteins) has been purified from ruminant placenta (1, 2). Some of them are detected in peripheral circulation of pregnant females, being used as a tool to investigate placental function in ongoing pregnancy and in obstetric diseases as well (3-8). The follow-up of trophoblastic function of ruminant species will be discussed below. Emphasis will be given to measurement of pregnancy-associated (-specific) proteins, but information related to P4 and ultrasound will be freely utilized in order to develop our comparative understanding of the monitoring of placental well-being.

GENERALITIES ON MONITORING OF PLACENTAL FUNCTION

A multiplicity of factors can influence concentrations of placental proteins (placental mass, blood flow through the intervillous space, compartmental distribution, half-life, assay characteristics, among others). Thus, any investigation on trophoblastic function requires a longitudinal follow-up during pregnancy period in order to pinpoint those pregnancies in which the fetuses have an increased risk of dying or developing pathologies. In veterinary medicine, ultrasound scanning and measurement of pregnancy-associated glycoproteins (PAG) are probably the best single tests available for pregnancy diagnosis. However, there is no single test currently available that immediately differentiates continuing from non-continuing pregnancies. Thus, at least two repeated scanning or blood sampling are necessary for effective prognostic use. Association of ultrasound examination, PAG and P4 concentrations has also proved to be of great interest for clinicians and researchers working on pathophysiology of pregnancy in ruminant species (7-9). In these species, the monitoring of placental function is relatively complex, requiring a multivariate analysis of concentrations of different hormones/proteins and the follow-up during a relatively long period.

PREGNANCY-MARKERS IN RUMINANT SPECIES

Pregnancy-associated glycoproteins (PAG), also known under a variety of other names including pregnancy-specific protein B (PSPB; (1)), pregnancy-serum protein 60 kDa (PSP-60; (10)) and SBU-3 antigen (11), were first described as placental antigens that were also present in the blood serum of the mother soon after implantation. They constitute a large family of placental glycoproteins synthesized by the mono- and binucleate trophoblastic cells (12-14). As revised by Sousa *et al.* (15), purified and semi-purified PAG preparations were used to immunize rabbits and the antisera obtained allowed the development of homologous (RIA-497) and heterologous RIA systems (RIA-706 and RIA-708).

PAG can be detected in maternal circulation of pregnant cows at days 28-30 of pregnancy (concentrations higher than 0.5 to 0.8 ng/mL, where 0.8 ng/mL constitute the threshold for positive pregnancy diagnosis). In early and mid gestation, maternal PAG concentrations increase slowly and gradually, remaining below than 160 ng/mL until day 240 of pregnancy. Around parturition, concentrations increase exponentially reaching peak values of 1,000 to 5,000 ng/mL only few days before delivery (6).

In pregnant ewes and goats, PAG can be detected at days 22-26 after breeding (16, 17). In these species, PAG profiles are quite different than those obtained in cattle. Concentrations increase faster from week 3 to 4, reaching higher levels during the first month of pregnancy (up to 20 ng/mL). Thereafter, concentrations remain elevated until parturition, no exponential increase being observed during prepartum period. During postpartum period, the rate of decline in PAG concentrations is faster in ewes and goats (4 weeks) than in cows (about 14 weeks).

MEASUREMENT OF PLACENTAL PROTEINS FOR FOLLOW-UP OF TROPHOBLASTIC FUNCTION

In veterinary practice, studies on trophoblastic function are scarce. They concern mostly physiologist and pathologist researchers who are interested in a better understanding of the different aspects involved in pregnancy failure. In the field, cost of analysis, as well as management related to serial blood sampling protocols in ruminants species are major limiting factors for follow-up of trophoblastic function.

Are levels of PAG useful to predict the outcome of pregnancy?

In cattle, the embryonic period extends from conception to the end of the differentiation stage (around day 42) while fetal period extends from day 42 to parturition (18). As reviewed by Hanzen *et al.* (19), most pregnancy losses occur during the early pregnancy period and are of non-infectious nature. However, the incidence of early fetal loss is increasing under the current intensive management systems, used specially for dairy cattle production (20, 21).

As described by Breukelman (22), PAG concentrations are significantly lower in animals in which pregnancy losses occur between day 26 and 120, compared to animals that abort during late pregnancy or that calve at term. These results agree with our clinical observations (unpublished data), in which at about 50% of pregnant cows having very low PAG levels are likely to experience pregnancy failure (data not published). In practice, due to the 50% possibility of having an alive newborn, the decision to intervene (interruption of pregnancy) is not as straightforward.

Interestingly, Lopez-Gatius *et al.* (23) recently described that the risk of fetal loss in high producing dairy cows (during the warm season) is 10 and 6.8 times more likely in cows with low (<2.5 ng/mL) or high (>4.0 ng/mL) PAG concentrations, respectively. These odds ratios were obtained in samples collected

at day 35 post-AI. It is noteworthy to remark that blood samples taken at different pregnancy periods (day 42, 49, 56 and 63) did not produce such high odds ratio.

Secretion of PAG when hostile uterine environment

In different studies, we could follow P4 and PAG concentrations in females in which embryo tried to survive in a hostile intra-uterine environment (7, 15, 24). In this approach, P4 gives information concerning corpus luteum (CL) function, while PAG reflects the trophoblastic secretory function of developing conceptus (5, 7, 17, 25). *Fig. 1* shows two distinct examples. The first female (heifer n. 0599; *Fig. 1A*) received a bovine embryo without having an active corpus luteum as revealed by the low P4 concentrations at any time of the venipuncture. This recipient had probably a low ovarian activity (low estrogen levels). In this condition, lack of P4 and low estrogens allowed the survival of the embryo in the uterus. PAG concentrations started to increase earlier, but during only a short period. Finally, the embryo could not survive, the PAG levels decreased and an estrus was clinically detected at day 56 after the reference estrus. The second cow (n. 3004; *Fig. 1B*) was inseminated close after parturition. PAG was still high at the time of insemination. After the PAG concentration decreased, P4 concentrations and ultrasound indicated a pregnancy status at day 28. At day 34, the cow was clearly not pregnant as indicated by very low PAG and P4 concentrations as well as by ultrasound examination.

The first example indicates that the lack of P4, even in a flaccid non estrogenic uterus does not allow the implantation of the embryo. The second example

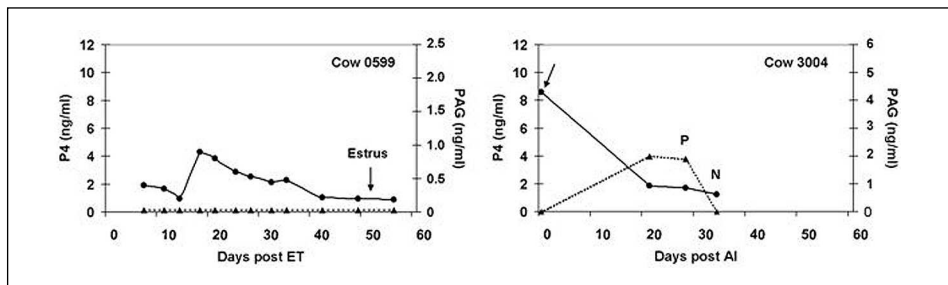


Fig. 1. Plasma PAG and P4 concentrations in two cows (n. 0599 and 3004) in which embryo survived for a limited time in unfavorable uterine environment. Dotted line represents the concentration of P4. Black and continuous line represents the PAG concentrations. Cow n. 0599 did not express detectable levels of P4, which indicate the lack of an active corpus luteum. However, an embryo transfer was performed at Day 7 after the sign of the last estrus. Around Day 18, a small peak was observed in the PAG concentrations (RIA-497). Thereafter, the PAG levels started to decrease till the end of the observation period. At Day 52 new signs of estrus were recorded. In cow n. 3004, Day 0 corresponds to day of the artificial insemination. A positive ultrasonographic pregnancy diagnosis (P) was followed by a negative pregnancy diagnosis (N) based on P4/PAG concentrations or by direct ultrasonographic observation of embryo. Arrows indicate the probable time of embryo death. (Adapted from Szenci *et al.* (7) and Perenyi (24)).

confirms that an insemination early after parturition is not suitable for a successful pregnancy due to the incomplete uterine involution.

Study of non-infectious embryonic/fetal mortalities

The follow-up of pregnancy by means of repeated ultrasonography, PAG and P4 measurements in maternal blood has also proven to be very useful to elucidate some physio-pathological conditions related to pregnancy failure in ruminant species. In 1998, Szenci *et al.* (7) described different situations in which concentrations of PAG allow a precise follow-up of trophoblastic function. Fig. 2 illustrates two interesting examples for veterinary practitioners. In the first cow (n. 3129; Fig. 2A), embryonic mortality occurred before day 50. At day 56, the P4 remained high while the PAG concentrations fell under the threshold, meaning the dead of fetus and placenta. The second cow (n. 3369; Fig. 2B) 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 P4 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 P4 concentrations. The second AI was probably responsible for the embryonic death occurring 45 days after the first AI.

PAG concentrations in recipients having received a somatic clone

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. In 1996, Ectors *et al.* (26) reported that concentrations of PAG are higher in maternal blood of recipient heifers carrying cloned embryos/foetus. The same authors described that abnormally high PAG

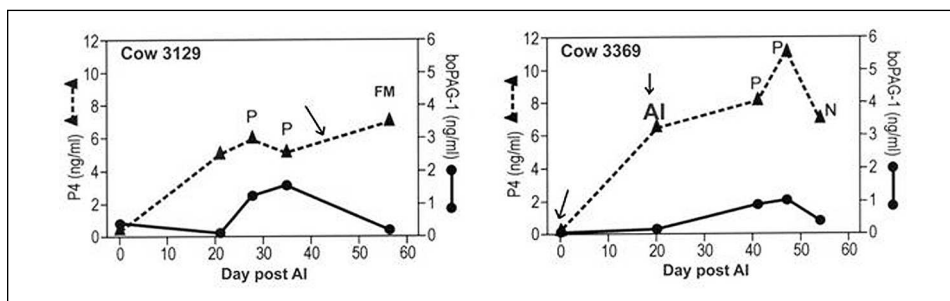


Fig. 2. Plasma PAG and P4 concentrations in two cows (n. 3129 and 3369) in which an initial positive ultrasonographic pregnancy diagnosis (P) was followed by a negative pregnancy diagnosis (N) based on P4/PAG concentrations or by direct ultrasonographic observation of fetal death. Day 0 corresponds to 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.* (7)).

concentrations in maternal circulation were observed in the presence of large amount of trophoblast tissues, as observed in hydatiform molar pregnancy. In the same way, concentrations of PSPB, were shown to be higher in maternal circulation of cows giving birth to a schistosomus reflexus calf (27).

More recently, by analyzing serial PAG determinations (RIA-497 and RIA-706) and successive ultrasound fetal measurements, Chavatte-Palmer *et al.* (28) demonstrated that cattle recipients carrying somatic clones show alterations in development speed as well as in PAG concentrations. PAG concentrations determined between days 34-36 and 50 of pregnancy are higher in cows carrying clone pregnancies that went to term than in control ones (*in vitro* produced embryos) (Fig. 3). Interestingly, in clone pregnancies, PAG concentrations determined by both RIA-497 and RIA-706 were lower in cows that experienced early pregnancy loss (between day 35 and 90) compared to cows that carried on pregnancy to a later date.

Concentrations of placental proteins in inter-specific pregnancies

As well, PAG levels are higher (about ten times) in recipient goats having received spanish ibex embryos (inter-specific pregnancies) than in those having

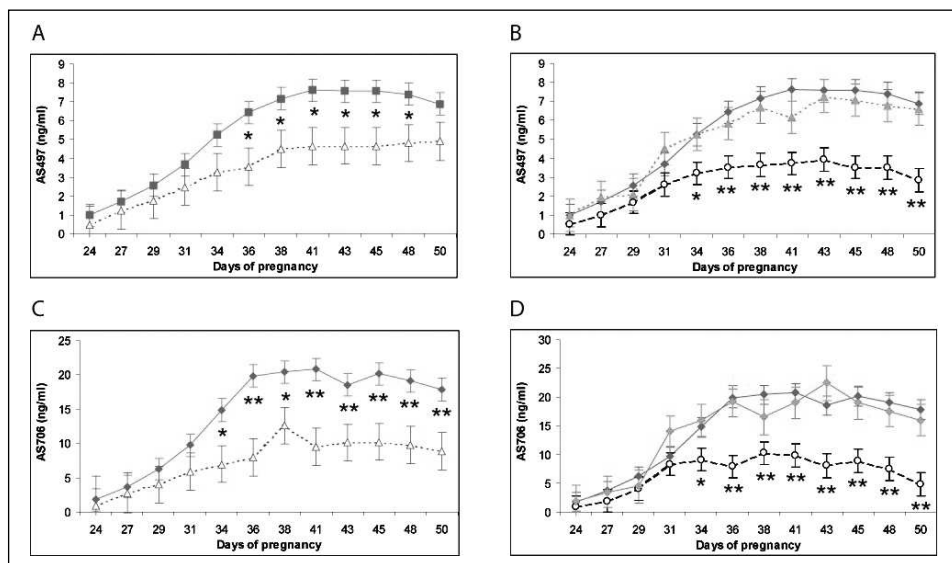


Fig. 3. Adjusted means (SD) for maternal PAG concentrations in cows that carried a control foetus (blue color), a clone that went to term (pink colour), a clone that had a late abortion (green colour) or a clone that suffered early foetal loss (white colour). Concentrations of PAG were measured by using RIA-497 and RIA-706. Graphics A and B: asterisks indicate a significant difference (* $P < 0.05$ and ** $P > 0.01$) between control and clone that went to term. Graphics C and D: asterisks indicate a significant difference (* $P < 0.05$ and ** $P > 0.01$) between the early foetal loss group and the two others. There was no significant difference between the cows that had late foetal loss and the ones that went to term. (Adapted from Chavatte-Palmer *et al.* (28)).

normal intra-specific gestations (29). In the same way, investigations realized in pregnant cows suggest a positive influence of both maternal breed and fetal genotype (race and sex) on peripheral concentrations of placental proteins. Mean peripartum PAG concentrations are higher in cows carrying a fetuses from distinct breeds than in those carrying fetuses from the same breed (6).

Study of embryonic/fetal mortalities due to infectious agents

Measurement of PAG/PSPB concentrations following infectious abortions in cattle (*Neospora caninum* and *Actinomyces pyogenes*) were described by different authors (24, 25). Semambo *et al.* (25) reported that sequential monitoring of PSPB identifies embryonic death when a continuing fall in plasma concentration is demonstrated. More recently, Zarrouk *et al.* (30) analysed PAG profiles in pregnant goats inoculated with *Toxoplasma gondii* or *Listeria monocytogenes*. Retrospective analysis of sequential measurements of PAG in goats also allowed for the determination of the onset of the disturbance of trophoblastic activity associated with death of one or multiple fetuses. In goats carrying a single fetus, PAG levels fall under the positive pregnancy threshold when the trophoblast dies, while in goats carrying 2 or 3 fetuses, analysis of the profiles clearly shows marked drops in concentration that can indicate when placental distress occurs (30, 31). Therefore, systematic application of this test in herds with a high rate of pregnancy failure can help to pinpoint phenomena which might be implicated in triggering these events.

Increased PAG concentrations in undernourished pregnant females

An association between severe energy or protein underfeeding and an increase in placental size has been well characterized in both human (32) and ovine species (33). Furthermore, an opposite association, between higher energy levels, reduced placental size and lower peripheral PSPB concentrations during gestation was recently demonstrated in ovine species (34). More recently, Sousa *et al.* (35) followed 11 pregnant Azawak zebu cows in Burkina Faso. Ten 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. These findings indicate that the PAG concentration can reflect different events and environmental conditions.

In conclusion, work on placental markers of trophoblastic function is in progress in ruminant species. Heretofore, the simultaneous use of ultrasound and the analysis of biochemical markers in blood samples could bring plentiful information on embryonic and fetal death as well as in miscarrying of pregnancy (ruminant females having fetal mortality, abortion, giving birth to stillbirth or to weak newborns).

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