

# Pregnancy – associated glycoproteins as a new diagnostic tool in cattle reproduction\*

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## Summary

Pregnancy-associated glycoproteins (PAGs) are powerful pregnancy markers in domestic cattle. These proteins are expressed in mono- and binucleate trophoblast cells from the first days of gestation until calving. Different molecules were identified as being expressed at various stages of pregnancy. However, up to date, their functions and activities during pregnancy were not yet established. Specific RIA tests were developed (classic and alternative RIA) and used to measure the concentration of these glycoproteins in blood during gestation and the postpartum period in cattle. In maternal blood, PAGs rise to detectable levels from days 24 to 28 after fertilization. A recent study indicated that PAGs can also be detected in milk samples. However, concentrations in milk are much lower when compared to those of plasma.

Keywords: pregnancy-associated glycoproteins, pregnancy markers, cattle, RIA

## Trächtigkeits-assoziierte Glykoproteine als neues Diagnostikum in der Rinderzucht

Trächtigkeits-assoziierte Glykoproteine (pregnancy-associated glycoproteins, PAG) sind bedeutungsvolle Marker zur Feststellung der Trächtigkeit beim Rind. Diese Proteine werden von speziellen Trophoblastzellen (ein- und mehrkernig) in den ersten Tagen der Trächtigkeit bis zum Abkalben gebildet. Während verschiedenen Zeitpunkten der Trächtigkeit werden sie als unterschiedliche Moleküle exprimiert, über deren Funktion und Wirkung bis heute wenig bekannt ist. Mit Hilfe von neu entwickelten spezifischen RIAs (klassische und alternative) können diese Glykoproteine im Blut während Trächtigkeit und Nachgeburtphase bestimmt werden. Die Konzentration von PAG steigt ab Tag 24 bis 28 nach der Befruchtung im mütterlichen Blut deutlich an und kann radioimmunologisch gemessen werden. Eine kürzliche durchgeführte Untersuchung zeigt, dass PAGs auch in der Milch nachgewiesen werden können, doch sind ihre Konzentrationen deutlich tiefer als im Blut der Mutter.

Schlüsselwörter: Trächtigkeits-assoziierte Glykoproteine, Trächtigkeits-Marker, Rind, RIA

## Introduction

The discovery of pregnancy-associated glycoproteins (PAGs) brought a revolution in cattle's reproductive techniques (Butler et al., 1982; Humblot et al., 1988; Zoli et al., 1991; Mialon et al., 1993). From a scientific and economic point of view it was a progression in the breeding of those animals. PAGs are synthesized in the outer epithelial cell layer (chorion/trophoblast) of the placenta (Beckers et al., 1998; Green et al., 1998; Garbayo et al., 2000). Part of the

molecules traffics into maternal blood and milk (Tainturier et al., 1996; Gajewski et al., 1999; Metelo et al., 2002; Lopez-Gatius et al., 2007; Gajewski et al., 2008a; Gajewski et al., 2008b). Previous investigations revealed the possibility of using RIA tests to measure the concentration of PAGs in those materials. The radioimmunoassay is a kind of method with very high specificity and sensitivity and can be used in routine pregnancy diagnosis in cattle.

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## Structural and biochemical differentiation in a range of PAG group

Pregnancy-associated glycoproteins have been discovered in the second half of the 20th century (Xie et al., 1991; Zoli et al., 1991). Since then many articles and publications about these proteins have been written and accordingly there appeared different names for PAGs. In the international literature PAG was described as: pregnancy-specific protein B (PSPB), pregnancy-specific protein 60 (PSP-60), or SBU-3 antigen (Humblot et al., 1988; Xie et al., 1991; Zoli et al., 1991; Zoli et al., 1992; Atkinson et al., 1993; Mialon et al., 1993; Xie et al., 1995). This large family of glycoproteins is expressed in the outer epithelial cell layer of the placenta of eutherian species. These molecules are synthesized by the mono- and binucleate trophoblastic cells and released into maternal blood starting from first weeks of pregnancy until delivery. PAGs are already detectable in placental tissue around the time of implantation (Beckers et al., 1999; Garbayo et al., 2000; Green et al., 2000; Wooding et al., 2005).

Variability of amino acid sequences, the carbohydrate component and the degree of glycosylation in several molecules result in great differences between various types of those glycoproteins (Zoli et al., 1991; Atkinson et al., 1993; Beckers et al., 1994; Klisch et al., 2006). Variable degrees of glycosylation correspond with plasma half life of PAGs including their peripheral concentration. A similar situation was observed in primates and equids with the existence of the more or less glycosylated forms of the human chorionic gonadotrophin (hCG) and the equine chorionic gonadotrophin (eCG). Another feature which characterizes pregnancy-associated glycoproteins is the molecular mass, which reaches the level of 70kDa, which is twice more than other plasma glycoproteins (Beckers et al., 1994; Patel et al., 2004; Klisch et al., 2006). Successive investigations revealed correlations between the level of PAGs in maternal blood and in milk. Recent tests demonstrated that the level of milk production can modify the concentration of PAGs circulating in blood. The more “liters of milk” is being produced, the less PAGs can be collected from maternal blood (Lopez-Gatius et al., 2007). At the same time the level of PAGs in milk is increasing (Tainturier et al., 1996; Gajewski et al., 2008a; Gajewski et al., 2008b).

Pregnancy-associated glycoproteins belong to a group of proteolytic enzymes, known as aspartic proteinases (AP). PAGs contain more than 50% of amino acid sequences identical to pepsin, cathepsin D and cathepsin E, but the vast majority of them are enzymatically inactive (Xie et al., 1991; Roberts et al., 1995; Szafranska et al., 1995; Xie et al., 1995; Perényi et al., 2002a). Only some of them can bind themselves to pepstatin A, which is an inhibitor of AP (Green et al., 1998; Green et al., 2000).

## The bovine PAG family

The first discovered bovine PAG was boPAG-1, also known as boPAG-67kDa (Green et al., 1998). The rest of the molecules belonging to this family got the following numbers as their names, which was accepted in the international nomenclature. So far recent investigations have proved the existence and sequences of 22 bovine PAGs, named appropriately boPAG-1 – boPAG-22. On the basis of biochemical analysis and biological properties of individual glycoproteins PAGs were partitioned into three groups:

- 1) The pepsinogen-like PAG (boPAG-8) group
- 2) The gonadotrophin-like PAG (boPAG-2) group
- 3) The major bovine PAG family (boPAG-1) group

### The pepsinogen-like PAG group

The boPAG-8 group is the family generally detected among animals which belong to Placentalia. Those specific peptides were also discovered in horses, cats, zebras, mice and rats (Green et al., 1998). In theory, these glycoproteins are enzymatically active, but so far this has not been scientifically confirmed (Szafranska et al., 1995; Beckers et al., 1999).

### The gonadotrophin-like PAG group

Several studies raised suspicions about LH-like activity substances existing in trophoblastic cells (Beckers et al., 1988; Beckers et al., 1994; Beckers et al., 1998). The following research on cows, in which the pituitary gonadotropic activity was reduced, demonstrated that the corpus luteum is still supported by additional luteotropic substances (Szafranska et al., 1995; Beckers et al., 1999). As revised by different authors, in 1950 Foot and Kaushik proved the presence of luteotropin-like activity in cotyledonary cells. It was a breakthrough discovery in cattle breeding. The next experiments using radioreceptor assay method made by Ailenberg and Shemesh (1953) showed that these molecules can stimulate the bovine granulosa cell layer to produce progesterone (Beckers et al., 1994; Beckers et al., 1998; Sousa et al., 2006). Other studies showed that the cDNA sequence of this molecule, named bovine chorionic gonadotrophin (Beckers et al., 1988), was closely related to the sequence of PAGs (Beckers et al., 1994; Beckers et al., 1998). The same team of scientists confirmed the theory about the similarity of properties of aspartic proteinases and luteinizing hormone (Beckers et al., 1998; Beckers et al., 1999). It is known that chemotripsin, also a proteinase, has the power to bind to LH-receptors and activate them (Sousa et al., 2006).

Until now the following boPAG-2 group's proteins were described: boPAG-11, boPAG-12, boPAG-13 (Green et al., 2000). The gonadotrophin-like PAGs are built from a polypeptide, which contains 372 amino acids (Beckers

et al. 1998; Beckers et al., 1999). Initially boPAG-2 is synthesized by trophoblastic cells as a 70kDa molecular mass protein, than is processed to smaller molecules (Beckers et al., 1994). Those molecules appear 17–19 days after fertilization, but they are detectable only in mono- and binucleated cells of the trophoblast (Beckers et al., 1998). So far, there are no tests, including RIA, which can detect PAG-2 in maternal blood at such an early time after insemination.

### The major bovine PAG family

The first discovered bovine PAG isolated from cotyledons was boPAG-67kDa, also known as boPAG-1. This group also contains the proteins: boPAG-56kDa, boPAG-67kDa, boPAG-75kDa (Klisch et al., 2006). The major bovine PAG family's molecules are expressed at the stage of early blastocyst to delivery and even a few days later (Green et al., 2000; Ushizawa et al., 2004). The synthesis in such early stage of pregnancy suggests that PAGs are needed in the implantation process and placentogenesis (Wooding et al., 2005).

An unexpected phenomenon occurred during research. Antigens, immunologically similar to boPAG-67kDa, were found in testicular tissue and in ovarian extracts. There is no confirmation that this interesting discovery has anything to do with PAG level in blood, and there are no other studies about it. However, we may presume, that there are more places, apart from the trophoblast, where PAG can be synthesized.

### Diagnostic methods

There are many methods being used to analyze the level of PAG (Butler et al., 1982; Humblot et al., 1988; Zoli et al., 1992; Mialon et al., 1993; Szenci et al., 1998a; Szenci et al., 1998b; Perényi et al. 2002b; Szenci et al., 2003; Gajewski et al., 2008a; Gajewski et al., 2008b). Ushizawa et al. (2004) proved that several PAG molecules are expressed at the very beginning of pregnancy, for example: days 7 to 14 (boPAG-11, boPAG-16, boPAG-17, or days 14 to 21 (boPAG-1, boPAG-5, boPAG-9). To proceed the research cDNA microarray analysis was used (Ushizawa et al., 2004). The glycoproteins were detectable around the time when the trophoblast forms definitive attachment to the uterine wall. Afterwards their concentrations increase gradually (Sasser et al., 1989; Zoli et al., 1992). However, most of those glycoproteins cannot be purified and are not yet available for radioimmunoassay and ELISA tests.

### Classical radioimmunoassay

In 1992, Zoli et al. researched PAG and measured its level by homologous PAG RIA. BoPAG-67kDa (Zoli et al. 1991) was used as a standard, tracer and immunogen to create antiserum (AS 497). The classical PAG-497 radio-

immunoassay gave the opportunity to measure PAGs in the blood of cows already at day 28 after insemination (Sousa et al., 2008) and between days 30 and 35 PAG can be detected in all cows with concentrations ranging between 0.5 and 0.8 ng/ml (Zoli et al., 1992). During the first months of gestation, concentration increases only slowly and gradually to levels of 160 ng/ml on day 240 after fertilization. Just before parturition the PAG concentration in maternal blood rises rapidly to 1–5 µg/ml and then decreases slowly after labor. The concentration becomes undetectable in all animals at about day 100 after delivery. The PAG level in maternal blood decreases only slowly after labor because peripheral blood concentrations are very high before delivery and the half-life of this glycoprotein is as long as 7–9 days (Sousa et al., 2003). Besides, the level of PAG in maternal blood can differ in several fetal genotypes like sex or breed (Zoli et al., 1992; Mialon et al., 1993). Investigations showed also that PAG concentrations observed in maternal blood were higher than in fetal serum (Zoli et al., 1992). This fact suggests that this glycoprotein is delivered preferentially into the maternal system.

### Alternative radioimmunoassays

Some years after classical PAG-497 radioimmunoassay was described, new experiments using RIA-706 and RIA-708 were performed (Gonzalez et al., 2000; Perényi et al., 2002b; Perényi et al., 2002c; Chavatte-Palmer et al., 2006). One of the reasons to test new antisera was the discovery of new pregnancy-associated glycoproteins. Different types of PAGs being temporally expressed in placental tissue and a high N-terminal amino acid identities in several species from which the molecules were taken initiated further research. PAG's molecules were isolated from the placenta of caprine, ovine and bovine species. The experiments showed the ability of different antisera to detect PAGs during early pregnancy in cattle (Gonzalez et al., 2000; Karen et al., 2003).

Obtained antisera against combinations of caprine PAG-55 + 62kDa or PAG55 + 59kDa (Garbayo et al., 1998) were used to examine bovine pregnancy. Bovine PAG was used as a standard and tracer, anti-caprine PAG55,59kDa as the first antibody (1:500,000; Gonzalez et al., 2000) and sheep anti-rabbit IgG as the second antibody for precipitation. The anti-caprine PAG55 + 59kDa cross-reacts with the plasma of cows, sheep and goats. New studies proved that this method of PAG measurement is more sensitive and the level of detected glycoproteins increases especially in the period from days 25 to 50 after insemination (Perényi et al., 2002c).

Studies by Lopes-Gatius et al. (2007) on Holstein-Friesian cows using RIA-497 (which is more effective than RIA 706) demonstrated that milk production is negatively correlated with the PAG concentration in maternal blood indicating a high transfer capacity of PAG from the maternal circulation into the mammary gland.

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Another finding reported by Perényi et al. (2002a) using RIA-497, RIA-706 and RIA-708 gave a new insight in the PAG's role in pregnancy. This report described changes in the level of PAG after embryo transfer in cows. The examination included also progesterone measurement. One embryo was transferred into the ipsilateral horn of the uterus. A retrospective analysis of the profiles after embryo transfer revealed the atypical case. Transferred embryos will survive if the corpus luteum is developed correctly but in this study one of the cows demonstrated a very low level of progesterone, what suggests very low activity of the ovary. This situation was a signal to intensify PAG synthesis and the concentration of PAG in blood increased in very early time of pregnancy. The embryo could not survive and the PAG concentration in blood slowly decreased. This case allowed to form a theory about the correlation between embryonic trophoblast formation and changes in the uterine wall.

### “Sandwich type” ELISA

Enzyme-linked immunosorbant assay “sandwich” type is often used to determine hormone or protein concentrations in a sample. It is also useful to determine PAG concentrations in Holstein cows and heifers (Green et

al., 2005). First, the plate is coated with captured monoclonal antibodies raised against semi-purified PAG molecules. Then, the sample is added and any antigen present binds to the captured antibody. Detecting antibodies are added next which will bind to the antigen. In this case it is polyclonal rabbit antiserum raised against PAG. Enzyme-linked secondary antibody is added and binds to the detecting antibody. With this technique PAG can be detected at about day 24 of pregnancy.

### Conclusion

In farm animals pregnancy diagnosis is one of the most important features. PAG measurement makes it possible to diagnose pregnancy at a very early stage. From 28 days after fertilization, the glycoproteins can be detected in maternal blood from most of the examined cows (30–35 days in all cows). PAGs also appear in the milk and can be measured by the same RIA methods like in blood. However, PAG concentrations in milk are only 4 to 16% of those measured in blood during early pregnancy. In later stages, the amount decrease to 0.6 to 2% of blood concentrations. Our studies confirmed the use of PAG RIA in blood for routine early pregnancy diagnosis.

### Glycoprotéines associées à la gestation: un nouveau moyen de diagnostic en élevage bovin

Les glycoprotéines associées à la gestation (pregnancy associated glycoproteins, PAG) sont des marqueurs importants pour le diagnostic de gestation chez les bovins. Ces protéines sont synthétisées par des cellules trophoblastiques spéciales (mono- et plurinucléées) durant les premiers jours de la gestation et jusqu'au vêlage. Suivant le stade de gestation, elles expriment diverses molécules dont la fonction et l'effet ne sont pas encore connues aujourd'hui. Ces glycoprotéines peuvent être mises en évidence dans le sang durant la gestation et la phase puerpérale au moyen de RIA développés spécifiquement. La concentration de PAG dans le sang maternel augmente significativement dès le 24<sup>e</sup>-28<sup>e</sup> jour après la fécondation et peut être mesurée radioimmunologiquement. Une étude réalisée récemment montre que les PAG peuvent aussi être mises en évidence dans le lait où elles sont toutefois en concentration plus faible que dans le sang.

### Glicoproteine associate alla gravidanza come nuovo mezzo diagnostico nell'allevamento dei bovini

Le glicoproteine associate alla gravidanza (pregnancy-associated glycoproteins, PAG) sono dei marcatori per confermare la gravidanza nei bovini. Queste proteine vengono formate da speciali cellule trofoblaste (con un o più nuclei) nei primi giorni di gravidanza fino alla filiazione. Durante vari momenti della gravidanza queste vengono espresse come molecole differenti la cui funzione non è finora molto conosciuta. Grazie ad un nuovo e sviluppato specificamente RIA (classico e alternativo) queste glicoproteine possono essere determinate nel sangue durante la gravidanza e nella fase postparto. Dal 24<sup>esimo</sup> al 28<sup>esimo</sup> giorno, la concentrazione di PAG nel sangue materno aumenta nettamente dopo l'inseminazione tanto da ottenerne una misura radioimmunologica. Effettuando una breve analisi si rileva che la PAG viene riscontrata pure nel latte ma a concentrazioni molto inferiori che nel sangue materno.

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