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VARIATIONS IN PLASMA MATRIX METALLOPROTEINASES AND PLACENTAL PREGNANCY-ASSOCIATED GLYCOPROTEINS DURING GESTATION IN RABBITS (*ORYCTOLAGUS CUNICULUS*)

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Abstract: Adaptation to pregnancy involves major maternal anatomical, physiological and metabolic modifications to support the mother's metabolic needs and those of the growing foetus. Invasion of embryonic territories and implantation in humans and other mammals is the result of an active biochemical process. The invasive faculty of cytotrophoblastic cells depends on their capacity to secrete proteolytic enzymes such as matrix metalloproteinases (MMPs). Many studies have addressed the variations in MMPs in placental tissues, but few have reported on their measurements in plasma. Furthermore, the Pregnancy-Associated Glycoproteins (PAGs), which are synthesized in the syncytiotrophoblast and used as early markers of pregnancy in domestic and wild animals, have not yet been studied in rabbits. In this study, we attempt to purify PAGs from rabbit placenta using a previously described methodology and to measure their concentrations as well as those of MMP-2 and MMP-9 in the plasma throughout pregnancy. In the course of the work, we noted certain modifications of the biochemical parameters in plasma during gestation, essentially in glycemia and lipemia. We detected proteins immunologically-related to PAGs in placental extracts between D_{14} to D_{21}. Until now, it has been impossible to develop a homologous RIA in order to measure PAGs in rabbit plasma, as the recovery of PAGs during the purification procedure was very low and PAGs were undetectable in rabbit plasma using heterologous RIAs. Finally, we demonstrated a rise in MMP-2 and -9 at the middle and the end of the gestation. However, the small variations presented by these matrix metalloproteinases preclude the use of plasma levels of these enzymes as early markers of pregnancy progress in the rabbit.

Key words: rabbit, pregnancy, MMPs, associated glycoproteins, genial tract.

INTRODUCTION

Adaptation to pregnancy involves major maternal anatomical, physiological and metabolic modifications to support the mother's metabolic needs and those of the growing foetus (Kalhan, 2000). It has been suggested that pregnancy is a complex physiological process similar to an inflammatory reaction (Bischof, 2001). Invasion of embryonic territories and implantation in humans and other mammals is not due to passive growth pressure but is an active biochemical process. The invasive faculty of cytotrophoblastic cells depends on their capacity to secrete proteolytic enzymes such as matrix metalloproteinases (MMPs), serine and cysteine proteinases (Sol-Church *et al.*, 2002). The MMPs play a role in normal and pathological processes, since they are collectively able to degrade the totality of extracellular matrix (ECM) components and to facilitate cell movements to

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various sites. Many studies have addressed the variations in MMPs in placental tissues of sheep (Salamonsen *et al.*, 1995; Riley *et al.*, 2000), cow (Zoli *et al.*, 1991), and monkey (Li *et al.*, 2003). The invasive process is regulated via a complex cooperation between cytokines, hormones, prostaglandins, enzymes and other factors, some of these factors being produced by the placenta (Keelan *et al.*, 2003). In contrast to pathological invasive phenomena, the processes of nidation and placentation are limited both in time (until mid-gestation) and in space (limited to the endometrium and proximal myometrium) (Bischof *et al.*, 2001). The spatio-temporal limitation of MMP activities is under the control of the tissue inhibitors of metalloproteinases (TIMPs) in concert with other uterine factors (glycoproteins of ECM), products secreted by endometrial cells (IGFPB-1, IL-1, IL-6, TNF-α, TNF-β) (Shimonovitz *et al.,* 1996; Bischof, 2001) and leptin produced by the feto-maternal unit (Masuzaki *et al.*, 1997). Maternal cells may also contribute to the control of endovascular cytotrophoblast invasion although the exact nature of the regulatory interactions between these cells is not fully understood (Campbell *et al.*, 2003).

For domestic animals, there are obvious economic advantages to being able to determine the pregnancy status of livestock as soon after mating as possible (Goel *et al.*, 1992). Proteins such as pregnancy-specific proteins (PSPs) A and B, secreted by the placenta can sometimes enter the maternal circulation and be useful indicators of pregnancy (Butler *et al.*, 1982). These glycoproteins, known as PAGs (Pregnancy-Associated Glycoproteins) since 1991, are used as markers and can be measured in the maternal blood of cow (Zoli *et al.,* 1991), goat (Garbayo *et al.*, 1998), sheep (El Amiri *et al.*, 2004) and pig (Szafranska *et al.*, 2003). The PAGs are members of a large family of aspartic proteinases, found in animals, plants, fungi and retroviruses. These PAGs have no lytic activity and show a large sequence identity with pepsinogens (Kageyama, 2002). These glycoproteins are produced by trophoblasts and are expressed by chorial epithelium of the placenta (Xie *et al.*, 1991). They have been extensively studied in domestic and wild ruminants (Zoli *et al.*, 1991; Beckers *et al.*, 1998; de Sousa Melo *et al.*, 2003, El Amiri *et al.*, 2004), but have not yet been studied in rabbits.

The aim of our study was to purify PAGs from the rabbit placenta and to measure these glycoproteins in the plasma as a function of the time of gestation. MMP-2 and MMP-9, the two MMPs which play a major role during nidation and placentation, were measured in the same plasma samples. Histological examination of the uterus and ovaries and the measurements of several biochemical parameters (glucose, total proteins, cholesterol and triglycerides) in plasma were also performed.

MATERIAL AND METHODS

Animals and experimental design

The study was carried out on a local strain of female domestic rabbits *Oryctolagus cuniculus*. Adult rabbits, weighing about 2 kg, were housed in the Biology Unit at the University of Algiers in accordance with the Home Office Regulations. Food and water were allowed *ad libitum*. The gestation period in the rabbit is 30 days. Ovaries and uterus were taken from 3 non-pregnant and 3 pregnant females 14 days *post coitum* (D_{14}) for histological studies. Pool of placentas are constitted for PAG purification, 8 placentas aged D_{18}, 5 aged D_{14}, 5 aged D_{16} and 5 aged D_{21}. To monitor gestation, females were weighed weekly.

Blood sampling

Several biochemical parameters were measured on plasma of 12 pregnant females. After 18 hours fasting, blood was drawn before mating (T_0), 4 days after (D_4), at weekly intervals thereafter (D_7, D_{14}, D_{22}, D_{30}) and finally at *post partum* (PP; 48 hours after the delivery), from the ear vein into containers with EDTA. Plasma obtained by centrifugation was stored at –20°C prior to analysis.

Glucose, total protein and lipid measurements in plasma

Cholesterol, triglycerides, glucose and proteins were measured in plasma using Randox kits (France) according to the method of Roeschlau *et al.* (1974), Trinder (1969), Barham and Trinder (1972) and Biuret (Henry *et al.*, 1974), respectively.

Quantification of matrix metalloproteinases MMP-2 and MMP-9 in plasma

MMP-2 (gelatinase A) and MMP-9 (gelatinase B) were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis SDS-PAGE gelatin zymography. Ten µl of diluted plasma (1/10 in PBS) were analysed by electrophoresis on 10% SDS gels containing 1 mg/ml of gelatin (Sigma, St Quentin-Fallavier, France) under non-reducing conditions, as previously described (Badier-Commander *et al.,* 2000). After electrophoresis, the proteins were renatured by replacing SDS with 2.5% Triton X-100, 2 x 30 min incubation, at room temperature, and the gels were incubated 19 hours at 37°C in 50 mM Tris-HCl buffer, pH 7.8 containing 10 mM $CaCl_2$. Gels were stained with 0.05% Coomassie Brillant Blue R-250. Gelatinolytic activities were quantified by densitometry using NIH image 1.60 ppc software. A standard sample was run on each gel and served as reference. The level of each MMP at each stage of pregnancy was expressed as a percentage of the T_0 level (time before pregnancy).

Organ collection

Rabbits were killed with an overdose of pentobarbitone (Rhone Merieux, Essex, UK). Ovaries, uterus, and oviducts were excised and fixed in aqueous Bouin solution for 48 hours, prior to embedding in paraffin for histology. Placental tissues were isolated, rinsed with isotonic solution and stored at –80°C prior to PAG purification.

Histology of reproductive tract

Five µm sections were obtained from paraffin-embedded ovarian, uterine and placental tissues with the micrometer (microtome American Optical, Michigan). Two staining procedures were performed on these sections: Masson's trichrome for general topography and collagens and Periodic Acid Schiff (PAS) to demonstrate glycoproteins (Martoja and Martoja, 1967).

Isolation and quantification of Pregnancy-Associated Glycoproteins (PAGs)

Proteins were extracted from placental tissues by homogenizing in saline buffer 0.1 M KH_2PO_4-K_2HPO_4, pH 7.6 (5 ml/g of tissue) in the presence of protease inhibitors (0.2M PMSF, 0.2% sodium EDTA) according to the method of Zoli *et al.* (1991). The homogenates were slowly shaken overnight at 4°C. After centrifugation, proteins from the supernatant were successively precipitated with 40% and 80% ammonium sulfate. In order to isolate PAGs, proteins were purified by ion exchange chromatography on DEAE cellulose eluted with a gradient of increasing NaCl concentration (0-0.1M) (Othmani-Mecif *et al.*, 2002). After dialysis, the fraction with the highest PAG content was submitted to high performance liquid chromatography (HPLC) on resin mono S (Waters 650, Millipore, MY). A gradient of NaCl (0-1M) was applied at a low flow (2 ml/min). The eluted fractions were submitted to radioimmunoassay. The measurement of PAG in each eluted fraction was carried out using four different immune sera and pure bovine PAG (Zoli *et al.*, 1992) as radiolabelled protein. The four immune sera were prepared against ovine PAG (oPAG 495, 1/ 20 000), bovine PAG (bPAG 497, 1/ 200 000) and caprine PAG I and II (cPAG I, 707, 1/150 000 and cPAG II, 708 1/40 000) by the Laboratory of Physiology of Reproduction, FMV, Liege, Belgium. Total proteins were determined by the method of Lowry, with the fraction V of BSA as standard. The ratio of immunoreactive substance/total proteins was calculated in each eluted fraction.

Data analysis

Results are expressed as means ± standard error. Data were analysed using Student's unpaired *t*-test. Significance was accepted when $P<0.05$.

RESULTS

Evolution of body weight of pregnant rabbit

The pregnant female showed a progressive increase in body weight (T_0: 1880 ± 410 g, D_{14}: 2480 ± 470 g, D_{30}: 2710 ± 470 g). This increase was in relationship with the size of the litter, the weight rise corresponding to approximately 50 g per kit. After parturition, the female did not immediately recover her initial weight (PP: 2580 ± 380 g).

Histological modifications of rabbit reproductive tract during pregnancy

Primary follicles were rare in pregnant rabbit ovary (Figure 1b). The corpus luteum is maintained throughout gestation, a characteristic that differentiates rabbits from other mammals. This structure presented large sized cells, the luteal cells of the granulosa and cells of reduced size corresponding to the internal theque. The uterus of the non-pregnant rabbit (Figure 1c) presented some folds in the endometrial mucosa; the myometrium is composed of two muscular layers, the internal layer being thicker than the external one. Villosities appeared in the functional endometrium of pregnant uterus and the glandular epithelium was intensely stained (Figure 1d). This epithelium showed a high activity (secretion in apex) (Figure 1e). The zone of nidation was surrounded by an alveolated territory (intervillous spaces). The embryo and its appendices were covered by the decidual layer, which isolated it from the uterine cavity. At high magnification, the feto-maternal junction (Figure 1g) showed syncytiotrophoblast cells with numerous nuclei in direct contact with the basal deciduous, which contained collagens (stained green by Masson's coloration).

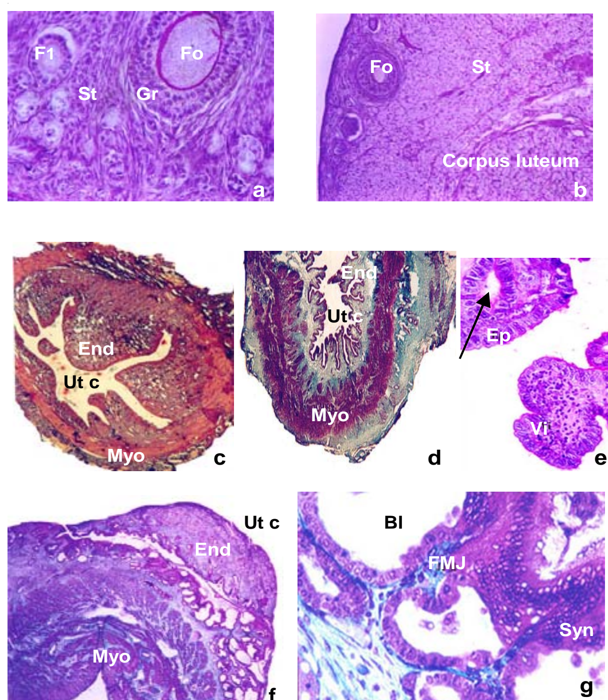


Figure 1. Histological observations of the ovaries (a, b, PAS staining) and uterus (c, d, e, Masson's trichrome) from non-pregnant (a, c) and pregnant (D_{14}; b, d, e) female rabbits. Histological observations of placenta from the D_{14} pregnant females are presented on figures f and g (Masson).

Ep: epithelium, FMJ: feto-maternal junction, End: endometrium, Fo: follicle, F_1: secondary follicle, Gr: granulosa, Bl: blood lacuna, Myo: myometrium, St: ovarian stroma, Syn: syncytotrophoblast, Ut C: uterine cavity, Vi: villosity, Epithelial gland.

Original magnification: a, b: ×25, c, d: ×10, e, g: ×40, f: ×4. (colour photographs are online available at http://www.dcam.upv.es/wrsa/wrs)

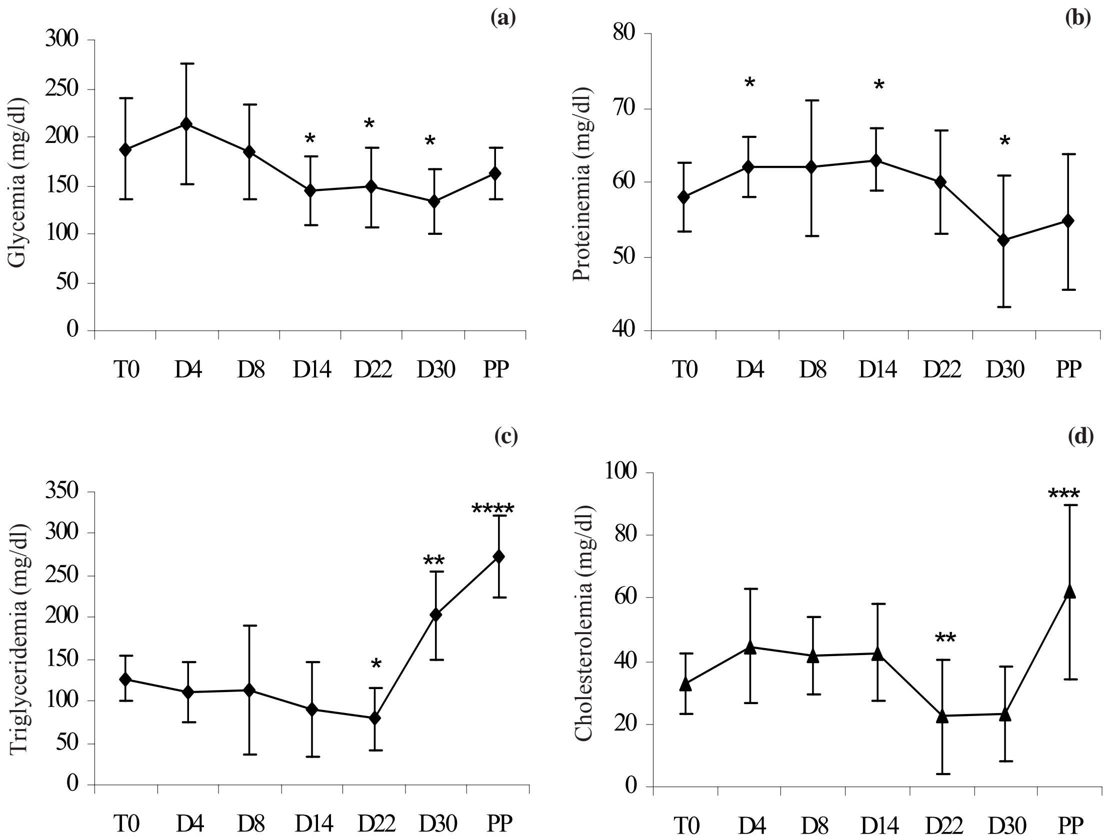
Biochemical plasma parameters during pregnancy

The glycemia of the non-pregnant rabbit (T_0: 187.5 ± 51.7 mg/dl) was in accordance with data reported by Vaissaire (1977). During pregnancy, glycemia decreased significantly at mid-gestation, decreased further before parturition (D_{30}) and increased after parturition without reaching the T_0 value (Figure 2a). Proteinemia increased slightly but significantly at the beginning of gestation and decreased at D_{30} before parturition (Figure 2b). Measurements of lipid parameters showed a slight decrease in triglyceridemia as well in cholesterolemia at D_{22} of gestation followed by a marked increase (P< 0.01) for triglycerides at D_{30}. Triglyceridemia was 126.6 ± 27.0 mg/dl at T_0, 78.9 ± 36.7 mg/dl at D_{22} and 202.5 ± 52.7 mg/dl at D_{30}.(Figure 2c). Cholesterolemia was 32.7 ± 9.7 at T_0 and 22.3 ± 12.4 mg/dl at D_{22} (Figure 2d). After parturition, the two measured lipid parameters significantly increased (Figures 2c and 2d).

Purification of PAG from placenta and its detection in plasma

PAGs were purified from a pool of D_{18} placentas using a previously described methodology. Detection of PAGs in crude extract, 40% SAS, 80% SAS precipitates and each fraction eluted from DEAE

Figure 2. Evolution of biochemical parameters in plasma during pregnancy in the rabbit. T_0: before pregnancy, PP: post partum, 48 hours after parturition. The *t*-test is evaluated at each age of pregnancy *vs*. D_4, considered as the initial pregnancy stage. (*, P<0.05; **, P<0.01; ***, P< 0.001; ****, P<0.0001)



cellulose was performed using four available immune sera. More immuno- reactive proteins were detectable in all tested samples using immune sera against ovine or bovine PAG (Figure 3). We concluded that immune serum against ovine PAG was the most sensitive for the detection of rabbit PAGs. Only a small fraction of PAGs from D_{18} placenta was detectable in the pass-through eluate of the ion exchange chromatography, indicating that the major part of these proteins were anionic at pH 7.6 and bound to the resin. The greatest quantity of immuno-reactive proteins was eluted with 0.04M NaCl (Figure 3). During the purification of PAGs from placentas of different gestation stages on DEAE cellulose, modifications of the elution pattern were observed: a greater quantity of PAGs from D_{14} placentas were eluted at a lower concentration of NaCl (0.02M) than PAGs from D_{16}, D_{18} and D_{21} placentas.

According to the results obtained during the procedure of purification of PAGs, the immune serum against ovine PAGs was used in order to detect these specific proteins in rabbit plasma. It was not possible to detect immunorective PAGs in rabbit plasma, whatever the stage of pregnancy.

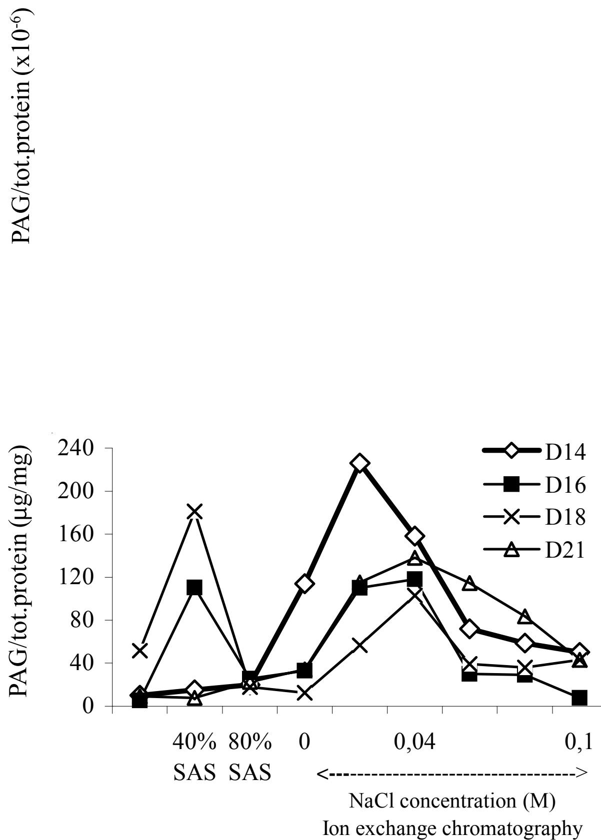


Figure 3. Purification of PAGs from rabbit placenta. PAG detection in crude extract, 40% SAS precipitate, 80% SAS precipitate and fractions eluted from DEAE cellulose of D_{18} placenta extract using four immune sera against bovine, ovine and caprine PAG, of D_{14}, $D_{16,}$ D_{18} and D_{21} placenta extracts, using immune serum against ovine PAG. The proteins of D_{14} placenta eluted from DEAE cellulose using 0.02M NaCl were further purified by HPLC on mono S resin.

Quantification of matrix metalloproteinases MMP-2 and MMP-9 in plasma during pregnancy

Gelatin zymography of plasma showed bands corresponding to gelatinolytic activities of proMMP-9-lipocalin complexes (MW of 115 kDa), proMMP-9 (MW of 92 kDa), active MMP-9 (MW of 75 kDA) and proMMP-2 (MW of 72 kDa). The gelatinolytic activity of proMMP-9-lipocalin complexes was not significantly modified during pregnancy except at the beginning (D_4) (Figure 4a). The level of proMMP-9 significantly increased at D_{14} (P<0,01) (Figure 4b), but this elevation was not observed for the active form (Figure 4c). In comparison with the basal state (T_0, non-pregnant), the two forms of MMP-9 presented a slight and significant increase at the end of pregnancy (D_{30}) (Figures 4b and 4c). Only the proform of MMP-2 was detectable in plasma. Measurement of this proMMP-2 activity during and after pregnancy showed that proMMP-2 existed at all stages of pregnancy and increased significantly at D_{14} and just before parturition (Figure 4d). No significant variation in the total MMP-9/total MMP-2 ratio was observed during gestation except after parturition (Figure 4e).

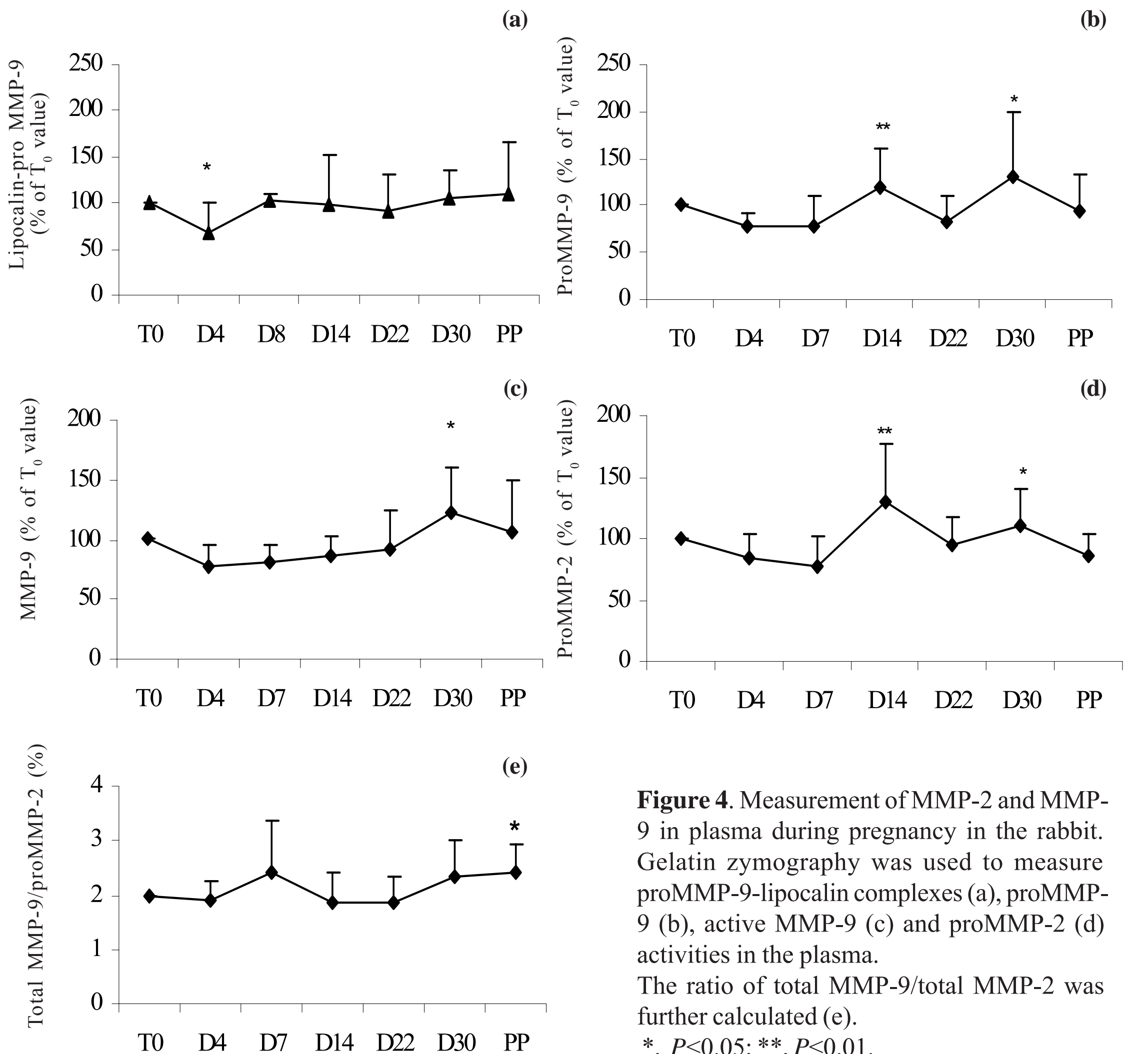


Figure 4. Measurement of MMP-2 and MMP-9 in plasma during pregnancy in the rabbit. Gelatin zymography was used to measure proMMP-9-lipocalin complexes (a), proMMP-9 (b), active MMP-9 (c) and proMMP-2 (d) activities in the plasma.
The ratio of total MMP-9/total MMP-2 was further calculated (e).
*, P<0.05; **, P<0.01.

DISCUSSION

In this study, we demonstrated certain modifications of the biochemical parameters in plasma during gestation in the rabbit, notably glycemia and lipemia. We also noted a rise in MMP-2 and -9 at the mid-stage and end of pregancy. Using previously described methodology, only small quantities of PAGs could be purified from rabbit placenta and no PAGs could be detected in plasma.

Biochemistry of pregnancy

Gestation was monitored by measuring body weight once a week. Plasma triglycerides (TG) decreased at D_{22} then increased at D_{30} and even more after parturition. For Wells *et al*. (1999), this parameter measured in the plasma of New Zealand female rabbits increases at D_{19} and decreases until D_{28} whereas Montoudis *et al.* (1999) demonstrated a reduction in plasma TG content at D_{22} followed by an increase at the end of gestation. Cholesterol decreased significantly at the 22nd day of gestation and it increased in post-partum plasma. The totality of free fatty acids and 50% of lipids required by the foetus are drawn by the placenta from the maternal circulation (Herrera, 2000). This could explain the decrease in lipid content in maternal plasma at D_{22} of gestation. The increase in plasma TG and cholesterol after parturition is probably related to lactation. Small variations in proteinemia could be detected: it increased meaningfully at D_{14} and decreased at D_{30}. The significant decrease in glycemia at the end of pregnancy is explained by the absence of food intake just before delivery (Lebas, 1999).

Pregnancy Associated Glycoproteins in rabbit

There are several methods for pregnancy diagnosis (Zoli *et al*., 1991; Butler *et al*., 1982), but only a few of them are useful in detecting early pregnancy. Pregnancy-Associated glycoproteins, PAGs, are secreted by trophoblastic mono- or binucleate cells (Szenci *et al*., 2003), are detectable in the maternal blood around the time of definitive attachment of the fetal placenta when the trophoblastic binucleate cells start to migrate and fuse with the endometrial cells forming the feto-maternal syncytium, releasing their secretory granules by exocytosis directly into maternal blood (Wooding, 1992). Using homologous or heterologous radioimmunoassays (RIAs), PAGs were measured in plasma for pregnancy diagnosis in various domestic and wild ruminants (Sasser and Ruder, 1987; Zoli *et al*., 1992; Willard *et al*., 1995). These RIA tests were also used to recognize the sex of the foetus (Ranilla *et al*., 1994) and single or twin pregnancies (Willard *et al*., 1995). For Karen *et al.,* (2003) the advantage of the PAG test over the progesterone test is that it can differentiate between pregnancy and prolonged inter-estrus intervals. During pregnancy, ovine and bovine PAGs exhibit spatially and temporally distinct expression patterns (Green *et al.,* 2000), and their concentration decreases rapidly after lambing, reaching basal levels (Ranilla *et al.,* 1994). In the same species, there are several PAGs which differed in amino acid sequence and apparent molecular mass (Garbayo *et al*., 1998) and each PAG presents several isoforms with different isoelectric points (El Amiri *et al*., 2004).

The present study consisted of purifying PAGs from rabbit placenta and of carrying out a PAG-RIA test in view of pregnancy diagnosis in the rabbit. We detected substances immunologically-related to PAGs in extracts of rabbit placenta eluted from ion exchange columns with 0.02M to 0.08M NaCl, whereas, for Garbayo *et al*. (1998), the interesting fractions in goat placenta were eluted between 0.04M and 0.08M NaCl. The largest quantity of PAGs from D_{14} placenta were eluted with 0.02M NaCl wheareas the largest quantity of PAGs from D_{16}, D_{18} and D_{21} placenta were eluted with 0.04M NaCl. The variation in the elution pattern according to the stages of pregnancy showed the existence of several PAGs or several isoforms of the same PAG with different isoelectric points and confirm the variable spatio-temporal expression demonstrated by Xie *et al*. (1997). The fact that the PAGs of the female rabbit are poorly recognized in heterologous RIAs raises some questions. Either PAGs are expressed at a very low level in rabbit tissue or PAGs are not expressed in rabbit placenta and the

protein recognized by the antibodies is pepsinogen F. Pepsinogen F is synthesised by rabbit foetal stomach and presents analogies of sequence with placental PAGs (Atkinson *et al*., 1993; Kageyama 2000). This fetal pepsinogen is synthesised early (Kageyama 2002) and is temporally regulated; this could explain the low reaction obtained with immune-serum antiPAG in the advanced stages of pregnancy. In order to avoid any interaction with this active aspartic proteinase, it would be of interest to extract PAGs from rabbit placenta at an earlier time of gestation (8 to 10 days).

PAGs remained undetectable in plasma throughout pregnancy whatever the type of anti-PAG immunoserum used (anti-oPAG, anti-bPAG, anti-cPAG I and II). It would be desirable to measure rabbit PAG with a different heterologous RIA system using a more specific immune serum such as anti-boPAG-9. PAG-9 is produced by binuclear invasive cells and is considered by Green *et al*. (2000) as the most extensively expressed PAG in the beginning of pregnancy.

Until now, it has been impossible to develop a homologous RIA in order to measure PAGs in rabbit plasma as the recovery of PAGs during the purification procedure was very low.

Evolution of MMP-2 and -9 in plasma of pregnant rabbit

Histological evidence of uterine tissue remodelling was observed at day 14. Invasion of the trophoblast into maternal tissue is an active biochemical process in which serine proteases, cathepsins and MMPs have all been implicated. The expression of most MMPs is normally low in reproductive tissues and is induced when ECM remodeling is required, such as during implantation and placentation (Bischof *et al*., 2003). Most MMPs, such as MMP-2 and -9, are secreted as inactive proenzymes by cytotrophoblast cells (CTBs) that are present in the decidua, the intima of the uterine spiral arteries and the proximal third of the myometrium. The MMPs produced by CTBs are capable of digesting the major constituents of the endometrial extracellular matrix (Bischof, 2001). The activity of these MMPs is regulated by many factors, such as TIMPs, that are secreted by the CTBs themselves (Librach *et al.,* 1991). The decidual (endometrial) cells and especially the large granular lymphocytes, LGL (Shi *et al.,* 1995) also produce MMPs. Furthermore, endometrial cells and decidual macrophages induce, via the production of TNF-α, the secretion of MMP-9 but not MMP-2 by the trophoblast (Meisser *et al.*, 1999).

Many studies on MMPs have been carried out on reproductive tissues but few have looked at plasma during pregnancy (Zucker *et al.*, 1992; Makrakis *et al*., 2003). MMPs synthesized in tissues seep into the maternal bloodstream, since the rabbit placenta is of the hemochorial type. Our results showed the presence of the latent form of MMP-2 in plasma before and throughout pregnancy. This result confirms the observations of Devlieger *et al*. (2000), who detected the latent form of MMP-2 in all gestational compartments at D_{18}, D_{23} and D_{30} of pregnancy in rabbit. This MMP-2 is probably produced by the extravillous CTB, although it is also strongly expressed in the uterus during the early phase of decidualisation and neovascularisation of the mouse placenta (Das *et al.*, 1997). Our results show that MMP-2 levels in plasma do not change during the first half of pregnancy. The increase in proMMP-2 level recorded in our samples at D_{14} could be due to a production by the blastocyst and annexes (Devlieger *et al*., 2000) and by the luteal cells, since, recently, Zhang *et al.* (2005) showed that MMP-2 is also produced by large luteal cells and endothelial cells. For Zucker *et al.* (1992), the level of MMP-2 in plasma increased by 50% during the second phase of human gestation; our study showed an equivalent increase of this enzyme, but essentially at mid- pregnancy. The endometrium contains leucocytes which produce TNF and IL-1b that stimulate the synthesis of this collagenase A by fibroblasts. (Shi *et al.*, 1995).

No active MMP-2 was detected in rabbit plasma during gestation. This could be due to the inhibition by progesterone of proMMP-2 activation by membrane-type 1 MMP (Zhang *et al.*, 2000).

ProMMP-9 showed an evolution analogous to that of proMMP-2 with a rise at the middle and at the end of pregnancy, as was previously demonstrated by Imada *et al.* (2002). The increase in MMP-2 and -9 noted at D_{14} could represent the tips of the iceberg. Perhaps they rise between D_7 and D_{14}, i.e. during implantation and placentation, then at D_{14}, the placenta is well formed, so daily measurements of MMP-2 and -9 between D_7 and D_{14} could be of interest.

Active MMP-9 did not change with advancing gestational age, as mentioned by Athayde *et al.*(1998). The elevated level of active MMP-9 produced by CTBs during the trophoblastic invasion phase in different species (Librach *et al.*, 1991; Wang *et al.*, 2001) are not apparent in the plasma since our samples show no variation in MMP-9 levels at the beginning of pregnancy.

At the end of gestation, we detected increases in both pro-MMP-2 and pro-MMP-9. We also recorded, at the end of pregnancy, a rise in active MMP-9, which plays an important role in the detersion of the placental tissues (Xu *et al.*, 2002). This increase in pro-MMP-2 and pro-MMP-9 in plasma can be linked to the decrease in progesterone in tissues and plasma. Indeed, progesterone secreted by the syncytiotrophoblast (STB) in human, downregulates the production of MMP-9 at the beginning of pregnancy (Shimonovitz *et al.*, 1998). In rabbit, progesterone is also produced by the corpus luteum (CL), an endocrine organ of transition, which is maintained throughout gestation. The expression of several MMPs in the human endometrium is controlled ultimately by ovarian steroids; progesterone acts to suppress endometrial MMP expression (Dong *et al.*, 2002).

At the end of gestation, progesteronemia decreases and the inhibition exerted on all proMMPs is eliminated (Zhang *et al.,* 2000, Imada *et al.*, 2002), then proMMP-2 and proMMP-9 productions increase, which would explain the rise of these pro-enzymes shown by our results at D_{30}.

Finally, at term of pregnancy, MMP levels correlated with those of leptin (trophoblastic origin) which stimulates production of MMP-2 (Castelluci *et al.*, 2000). The ratio MMP-9/ proMMP-2 in our samples rises significantly after parturition, as progesterone decreases and endometrial remodelling begins.

CONCLUSION

Currently, no known link exists between the PAGs, glycoproteins produced notably by the placenta and used as markers of early pregnancy in various species (Beckers, 1999) and MMP-2 and -9, proteolytic enzymes produced by endometrial, trophoblastic and luteal tissues; both PAGs and MMPs are proteinases, the former is devoid of lytic activity and the latter can degrade most components of ECM. Although the presence of PAGs in rabbit placenta remains open to controversy, it is nevertheless interesting that we observed in mid-gestation a simultaneous rise in circulating levels of a PAG-like compound and of MMP-2 and -9. However, the small variations presented by these matrix metalloproteinases preclude the use of these enzymes as early indicators for the follow-up of pregnancy. The studied MMPs cannot be proposed as an alternative to the measurement of hormones, such as progesterone.

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