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Effect of early pregnancy on the expression of progesterone receptor and progesterone-induced blocking factor 1 in ovine liver

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Abstract: Liver plays important roles in the innate and adaptive immunity, and contributes to the maternal immune adjustments during pregnancy. Progesterone (P4) has key effects on immunomodulation of the maternal uterus during pregnancy. In this study, livers were obtained at day 16 of the estrous cycle and at days 13, 16 and 25 of pregnancy (n = 6 for each group) in ewes. The effects of early pregnancy on the expression of P4 receptor (*PGR*) and progesterone-induced blocking factor 1 (*PIBF1*) were analysed through RT-qPCR assay, Western blot and immunohistochemistry analysis. Our results showed that the isoforms of PGR with molecular weights of approximately 60 kDa (PGR60) and 89 kDa (PGR89) were strongly expressed in the livers from pregnant ewes ($P < 0.05$), but there was no expression of the isoform of PIBF1 with a molecular weight of approximately 55 kDa (PIBF55) on day 13 of pregnancy. The PGR protein was mainly limited to the endothelial cells of the proper hepatic arteries and portal veins, and hepatic cells. In conclusion, the PGR89 and PGR60 were up-regulated, and PIBF55 was down-regulated on day 13 of pregnancy, which may be involved in maternal hepatic immunoregulation during early pregnancy in sheep.

Keywords: ewe; immunoregulation; isoform; progesterone

Progesterone (P4) is mainly produced by the corpus luteum (CL), and is an important endogenous steroid in the regulation of estrous cycle, pregnancy, and embryogenesis in humans and other species (Micks et al. 2015). During early pregnancy in ruminants, conceptus, including embryo and its associated extra-embryonic membranes, secretes interferon-tau (IFNT). IFNT is the primary pregnancy recognition hormone which acts on the uterus and/or CL to maintain P4 production by the ovarial CL (Spencer et al. 2016). It is through binding to P4 receptor (PGR), including nuclear PGR (PGR-A and PGR-B) and membrane PGR (mPGR), that P4 plays key roles in reproductive functions in cattle (Kowalik et al. 2013). Numerous target genes are transcriptionally activated by P4 through the binding of its specific nuclear receptors, which are implicated in the cell growth, development, differentiation, and homeostasis in the uterine endometrium (Simmen and Simmen 2002). Progesterone-induced blocking factor 1 (PIBF1) is involved in the immunologic and proliferative actions of P4 in U373 cells derived from a human astrocytoma grade III (Gonzalez-Arenas et al. 2014). PIBF1 changes cytokine ratios to decrease cell-mediated responses, and affects the helper T

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cell 1 (Th1)/Th2 balance during pregnancy in mice (Szekeres-Bartho and Wegmann 1996). Progesterone-treated lymphocytes or peripheral blood mononuclear cells (PBMCs) of healthy pregnant women release PIBF1 that blocks cytotoxic activity and prostaglandin F2α synthesis (Szekeres-Bartho et al. 1985a, b).

As a non-lymphoid organ, liver contains a substantial population of immunologically active cells, which play essential roles in innate and adaptive immunity (Parker and Picut 2012). Nuclear factor erythroid 2-related factor 2 (Nrf2) is essential for mediating maternal hepatic adaptive responses and adequately regulation of the number and size of maternal hepatocytes during normal pregnancy in mice (Zou et al. 2013). Hepatic cytochrome P450 2d mRNA, and activities of cytochrome P450 26a1 and retinoic acid receptor β are increased during pregnancy in mice (Topletz et al. 2013). The asialoglycoprotein receptor and the mannose receptor in the liver are induced by P4 during pregnancy in mice (Mi et al. 2014), and liver participates in maternal immune adjustments in mice. We hypothesise that early pregnancy exerts its effects on the expression of PGR and PIBF1 in ovine liver. Therefore, the objective of this study was to determine the expression of PGR and PIBF1 in livers from non-pregnant and early pregnant ewes, which may be useful to understand the maternal hepatic immune adjustments during early pregnancy in ruminants.

MATERIAL AND METHODS

Animals and experimental design. Twenty-four healthy multiparous Small-tail Han ewes (n = 6 for each group), 18 ± 2 months old, were housed in Hebei Province, China. All procedures were approved by the Hebei University of Engineering Animal Care and Use Committee, and humane animal care and handling procedures were followed throughout the experiment. All fertile matings occurred after the detection of estrous behaviour (day 0), and there were three pregnant groups (days 13, 16 and 25 of pregnancy). Normal rams were replaced by vasectomized rams in the nonpregnant ewes (day 16 of the estrous cycle). Hepatic samples were collected at days 13, 16, and 25 of pregnancy, and at day 16 of the estrous cycle. The ewes were killed and the conceptuses in the uterus were examined for confirmation of pregnancy. Hepatic transverse pieces (0.3 cm^3) were fixed in fresh 4% (w/v) paraformaldehyde in phosphate buffered saline buffer (pH 7.4), and the remaining portions of hepatic samples were frozen in liquid nitrogen for subsequent reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blot analysis.

RNA extraction and RT-qPCR assay. Total RNA was extracted from the hepatic samples using TRIzol (Invitrogen, USA) according to the manufacturer's instruction. The absorbance at 260 and 280 nm, and ratio 260/280 were used to check the concentration and purity of RNA preparations, and the quality of RNA preparations was assessed according to bands of 28S and 18S rRNA through agarose gel electrophoresis. A FastQuant RT kit (Tiangen Biotech Co., Ltd., China) was used to reverse transcribe approximately 1 μg of the total RNA into cDNA. Primers (Table 1) were designed and synthesised by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China) and amplification efficiencies of primer sequences of *PGR, PIBF1* and glyceraldehyde phosphate dehydrogenase (*GAPDH*) were evaluated before quantification. The PCR fragments were sequenced to check for specificity. A SuperReal PreMix Plus kit (Tiangen Biotech Co., Ltd.) was used for RT-qPCR, according to the manufacturer's instruction. The $2^{-\Delta\Delta Ct}$ analysis method was used to quantify the relative expression values for RT-qPCR assay (Livak and

Table 1. Primer sequences for quantitative PCR

Gene	Primer	Sequence	Product (bp)	Accession No.
PGR	forward	CAACAGCAAACCTGATACCT	183	XM_012169084
	reverse	CCATCCTAGTCCAAATACCATT		
PIBF1	forward	CCAGGCAGCTAATTGAACGG	189	XM_004012184
	reverse	GGGCTAGTACCTGCTTCTGG		
GAPDH	forward	GGGTCATCATCTCTGCACCT	176	NM_001190390.1
	reverse	GGTCATAAGTCCCTCCACGA		

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Schmittgen 2001), with *GAPDH* as the reference gene to normalise the data, and the mixture of the four groups was used as the mean CT.

Western blot analysis. Hepatic protein samples were prepared by RIPA Lysis Buffer (BL504A; Biosharp, China), and a BCA Protein Assay kit was used to detect the concentrations of proteins (Tiangen Biotech Co., Ltd.). Protein samples were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride membranes (Millipore, USA). The membranes were incubated with primary antibodies at 37°C for 1 h, and the primary antibodies included a mouse anti-PGR monoclonal antibody (Abcam, UK; ab2765, 1 : 1000) and a rabbit anti-PIBF polyclonal antibody (Santa Cruz Biotechnology, USA; sc-99129, 1 : 1000). Secondary goat anti-mouse IgG-HRP (Biosharp, BL001A), anti-rabbit IgG-HRP (Biosharp, BL003A) were diluted at 1 : 2000 (37°C for 40 min). A Prolight HRP chemiluminescence detection reagent (Tiangen Biotech Co., Ltd.) was used to visualise protein signals. An anti-GAPDH antibody (Santa Cruz Biotechnology; sc-20357) was used to monitor the sample loading at a dilution of 1 : 1000. Quantity One V452 (Bio-Rad Laboratories, USA) was used to quantify the intensity of the blots, and the relative levels were calculated using GAPDH.

Immunohistochemistry analysis. The embedded hepatic samples were cut into sections (0.5 μm) and mounted on glass slides. The embedded sections were deparaffinised in xylene and rehydrated in ethanol, and were stained by hematoxylin and eosin (HE). Immunohistochemical localization for PGR in the hepatic tissue was performed using the mouse anti-PGR monoclonal antibody (Abcam; ab2765, 1 : 1000). For a negative control, non-immune goat serum was used in place of the anti-PGR antibody. The antibody binding sites in the tissue sections were visualised using a DAB kit (Tiangen Biotech Co., Ltd.). Finally, the images were captured using a light microscope (Nikon Eclipse E800, Japan) with a digital camera AxioCam ERc 5s, and the intensity of staining and density of the stained cells were analysed through the images. The immunostaining intensity of the different hepatic tissue samples from different ewes (n = 6 for each group) was rated by 2 different investigators in a blinded fashion, and histological subtypes were analysed by assigning an immunoreactive intensity of a scale of 0 to 3, as described in a previous report (Zhang et al. 2018). An intensity of 3+ was given to the cells with the highest staining intensity, and an intensity of 0 was assigned to the cells with no immunoreactivity.

Statistical analyses. The data for the relative expression levels of *PGR* and *PIBF1* mRNA, PGR and PIBF1 isoforms were analysed as a completely randomised design with six animals per group using the Proc Mixed models of SAS (Version 9.1, 2004). For livers from different stage of gestation or pregnancy status, the model contained the random effect of ewe and fixed effects of stage of gestation, pregnancy status and the interaction of stage of gestation and pregnancy status. The comparisons among the relative expression levels of different groups were performed using the Duncan method and controlling the experiment-wise type ± error equal to 0.05. Data are presented as Least Squares Means. $P < 0.05$ was considered to indicate a significant difference.

RESULTS

Relative expression levels of PGR and PIBF1 mRNA in the livers. The RT-qPCR assay revealed (Figure 1) that the relative expression level of *PGR* mRNA was the highest in the livers at day 13 of pregnancy among the four groups ($P < 0.05$), but there were no significant differences in the expression level of *PGR* mRNA between the ewes from days 16 and 25 of pregnancy ($P > 0.05$). Furthermore, the relative expression level of *PIBF1* mRNA was the lowest at day 13 of pregnancy ($P < 0.05$), and there were no significant differences in the expression levels of *PIBF1* mRNA among the non-pregnant ewes, and the ewes from days 16 and 25 of pregnancy ($P > 0.05$).

Expression of PGR and PIBF1 proteins in the livers. Western blot analysis revealed that the PGR proteins were expressed in the livers (Figure 2), and the isoforms of PGR with molecular weights of approximately 60 kDa (PGR60) and 89 kDa (PGR89) were strongly expressed in the livers at day 13 of pregnancy ($P < 0.05$; Figure 2). The expression of PGR60 and PGR89 in the livers from non-pregnant ewes was the lowest ($P < 0.05$).

It was shown in Figure 2 that the isoform of PIBF1 with a molecular weight of approximately 90 kDa (PIBF90) was expressed in the livers, but early pregnancy had no significant effects on the

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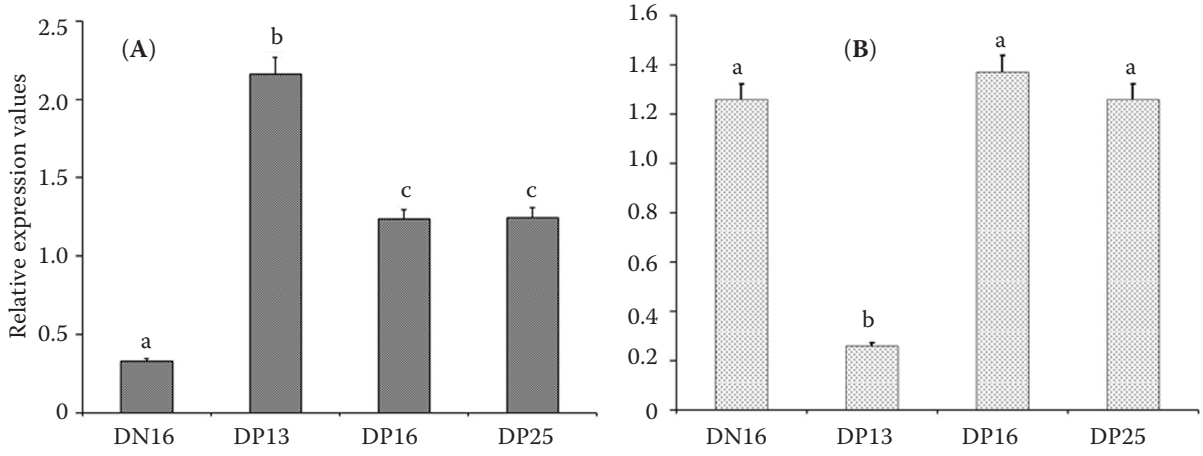

Figure 1. Relative expression values of *PGR* (**A**) and *PIBF1* (**B**) mRNA in the liver of non-pregnant and pregnant ewes

DN16 = day 16 of non-pregnancy; DP13, DP16, DP25 = day 13, 16, 25 of pregnancy

[a–c]significant differences ($P < 0.05$) are indicated by different letters within different bars

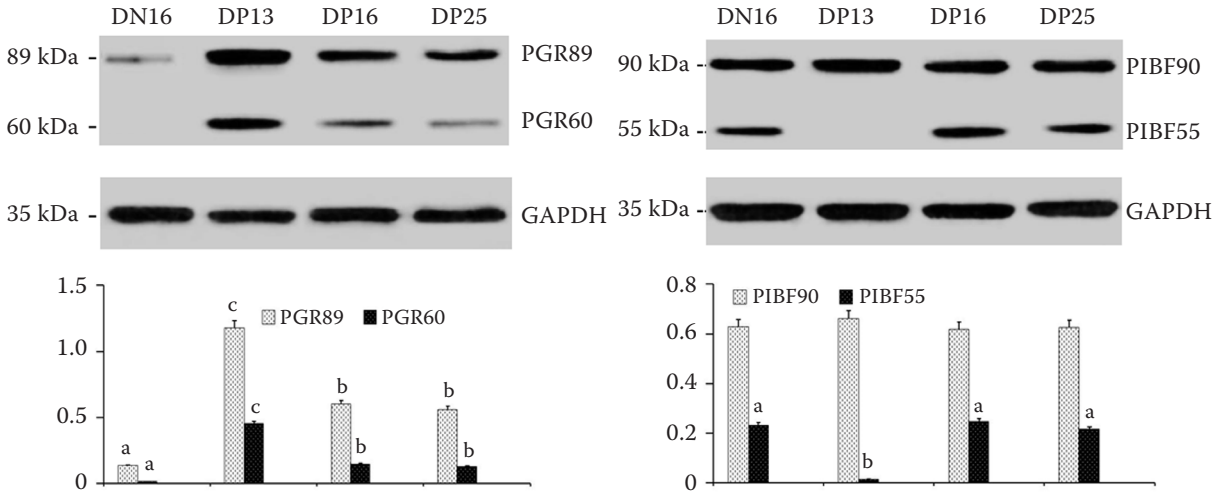

Figure 2. Expression of PGR and PIBF1 proteins in the liver of non-pregnant and pregnant ewes

DN 13, DN 16 = day 13 or 16 of non-pregnancy; DP13, DP16, DP25 = day 13, 16 or 25 of pregnancy; PGR60, PGR89 = isoform of PGR with molecular weight of approximately 60 or 89 kDa; PIBF55, PIBF90 = isoform of PIBF1 with molecular weight of approximately 55 or 90 kDa

[a–c]significant differences ($P < 0.05$) are indicated by different letters within the same colour bar

expression levels of PIBF90 ($P > 0.05$). Furthermore, the isoform of PIBF1 with a molecular weight of approximately 55 kDa (PIBF55) was not detectable in the livers at day 13 of pregnancy, and was expressed in the livers at day 16 of the estrous cycle, and days 16 and 25 of pregnancy, but there were no significant differences in the expression levels between the ewes at day 16 of the estrous cycle, and the ewes at days 16 and 25 of pregnancy ($P > 0.05$).

The immunohistochemistry for PGR protein in the livers. The antibody reacted with the different isoforms of PGR that existed in the tissue sections of livers. The PGR proteins were mainly limited to the endothelial cells of the hepatic arteries and hepatic portal veins, and hepatic cells (Figure 3). Furthermore, the staining intensity for PGR was 0, 1+, 3+, 2+, and 2+ for the negative control, the livers from day 16 of the estrous cycle, and the livers from days 13, 16 and 25 of pregnancy,

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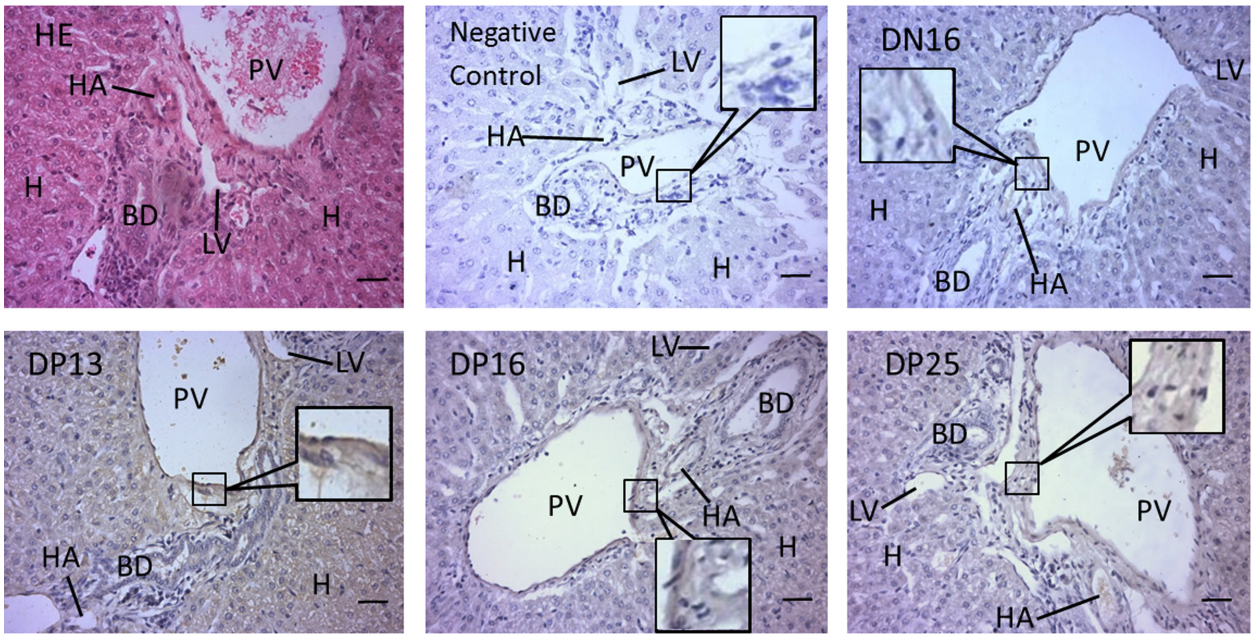

Figure 3. Immunohistochemical localization of PGR protein in the liver. Liver is divided into lobes, and a lobe is made up of hepatic lobules. A portal triad is a component of the hepatic lobule consisting of proper hepatic artery (HA), portal vein (PV), small bile ductile (BD) and lymphatic vessels (LV)

HE = stained by hematoxylin and eosin; H = hepatocyte; DN16 = day 16 of non-pregnancy; DP13, DP16, DP25 = day 13, 16 or 25 of pregnancy; bar = 50 μm

respectively (Figure 3). The staining intensity was as follows: 0 = negative; 1+ = weak; 2+ = strong; 3+ = stronger.

DISCUSSION

Maternal immune system must be adjusted by the conceptus in the uterus to avoid the immune destruction to the semiallogeneic fetus during early pregnancy in ruminants (Yang et al. 2014). It is known that P4 exerts essential effects on the immunomodulation of the maternal uterus and the immune system through an endocrine manner. There is a maternal tolerance of the fetus, which is necessary for successful pregnancy in mammals (Bonney 2017). Immune tolerance exists in the liver, which is mediated by the cellular mechanisms (Doherty 2016). Therefore, liver may be implicated in the maternal immune adjustments. In this study, a changed expression of PGR and PIBF1 in sheep livers during early pregnancy, indicating that early pregnancy exerted its effects on the maternal liver in sheep, was reported for the first time.

It is through binding to PGR that P4 regulates reproductive and immune functions. At different stages of early pregnancy, the expression of *PGR* mRNA and protein changes in the bone marrow (Zhang et al. 2017), spleen (Yang et al. 2018), lymph node (Yang et al. 2017) and thymus (Zhang et al. 2018) in sheep. It has been reported that endometrial *PGR* mRNA levels were decreased between days 1 and 11, and then increased between days 13 and 15 in cyclic ewes. Furthermore, *PGR* mRNA levels were increased between days 11 and 17, and decreased between days 17 and 25 in pregnant ewes (Spencer and Bazer 1995). Our RT-qPCR assay showed (Figure 1) that the relative expression level of *PGR* mRNA was significantly higher in the livers from day 13 of pregnant ewes, which indicated that P4 exerted its effects on the maternal liver. Therefore, P4 plays a role in regulating maternal hepatic function through an endocrine mechanism during early pregnancy in sheep.

P4 exerts its different effects through binding to functionally different isoforms of PGR (Lonard and O'Malley 2012), including nuclear PGR-A (81 kDa) and PGR-B (116 kDa) in the bovine myometrium (Slonina et al. 2012). PGR-C with a molecular weight of approximately 60 kDa is found in T47D cells, and primarily restricted to the cytosolic fraction rather than the nuclear fraction (Condon et

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al. 2006). The effects of P4 on the different tissues are changed with the expression ratio of PGR-B to PGR-A and/or PGR-C (Zakar and Mesiano 2011). Our Western blot assay revealed (Figure 2) that the PGR60 and PGR89 were strongly expressed in the livers from pregnant ewes ($P < 0.05$). Therefore, it is suggested that P4 mainly regulates the maternal hepatic immune function through up-regulation of PGR89 and PGR60 during early pregnancy in sheep.

P4 induces synthesis of PIBF1 in P4 receptor-positive T-lymphocytes. PIBF1 exerts its inhibitory effects on cell-mediated immune reactions, which results in a shift from type 1 to type 2 cytokines in the PBMCs of pregnant women (Raghupathy et al. 2009). Many PIBF1 isoforms are expressed in breast tumors and MCF-7 mammary carcinoma cells, such as the full-length PIBF1 (90 kDa), 34 kDa, 55 kDa, and 66 kDa variants, and different PIBF1 variants have different functional attributes (Polgar et al. 2003; Lachmann et al. 2004). Full-length PIBF1 (89 kDa) functions as a transcription factor to regulate cell cycle, whereas the smaller splice variants potentially act both as transcription factors and cytokines (Bogdan et al. 2014). PIBF1 expression is regulated by P4 through intracellular PGR, and PIBF1 is associated with the immunological and proliferative actions of P4. Our Western blot assay showed that PIBF55 was not detectable in the ovine liver on day 13 of pregnancy (Figure 2), which may be induced by up-regulation of PGR89 and PGR60 on day 13 of pregnancy. The isoform PIBF55 may have distinct immunological effects on the liver compared with those of the full-length PIBF. Therefore, P4 may exert its immunological and other actions through up-regulation of PGR89 and PGR60 and down-regulation of PIBF55 in maternal liver during early pregnancy in sheep.

The liver is divided into lobes, and a lobe is made up of hepatic lobules. A central vein is present in the middle of the hepatic lobule, and portal tracts are at its corners that contain interlobular branches of hepatic artery and portal vein, biliary ductules, lymphatic vessels and nerves (Kmiec 2001). Our immunohistochemistry results showed that the immunostaining for the PGR protein was limited to the endothelial cells of the proper hepatic arteries and portal veins, and hepatic cells (Figure 3). The staining intensity for the PGR was higher in the livers from day 13 of pregnancy than that from day 16 of the estrous cycle, days 16 and 25 of pregnancy (Figure 3). The portal veins and hepatic arteries merge upon and entry into the liver lobule at the portal field, and exit at the central vein (Kmiec 2001). Therefore, we suggest that the changed expression of PGR in the liver may be induced by early pregnancy through blood circulation in sheep.

CONCLUSION

The PGR89 and PGR60 were up-regulated in the liver on day 13 of pregnancy, but the PIBF55 was not detectable on day 13 of pregnancy. The PGR protein was limited to the endothelial cells of the proper hepatic arteries and portal veins, and hepatic cells. It is suggested that early pregnancy exerts its effect on the maternal liver through P4, which results in the up-regulation of PGR89 and PGR60 and down-regulation of PIBF55 on day 13 of pregnancy through blood circulation in sheep.

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