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THE QUANTITATIVE EXPRESSION OF PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR (PPAR) GENES IN PORCINE ENDOMETRIUM THROUGH THE ESTROUS CYCLE AND EARLY PREGNANCY

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Peroxisome proliferator activated receptors (PPAR) are a family of the nuclear receptors which play an important role as transcriptional factors. The aim of the present study was to determine the expression of PPARs (α, β, γ) mRNA in the porcine endometrium during the estrous cycle and periimplantation period. Gilts were divided into two groups (cyclic and pregnant), synchronized and superovulated. The animals from the first group were slaughtered throughout the estrous cycle: 2-4, 5-8, 9-10, 11-12, 13-15, 16-17 and 18-21. Gilts from the second group were inseminated and slaughtered at different days of pregnancy to create subgroups: 5-8, 9-10, 11-12, 13-15, 16-17, 18-21, 21-30. PPAR mRNA expression in the endometrium was analyzed by real-time PCRs. During the estrous cycle the expression of PPARγ1 mRNA was significantly higher on days 13-15 than at the remaining stages. The expression of PPARα and β transcripts showed a similar pattern and the lowest levels were observed on days 2-4, 16-17 and 18-21 in comparison with the remaining stages (days 5-8, 9-10, 11-12). During pregnancy a significant increase in the expression of PPARγ1 mRNA was noted on days 16-17, 18-21 and 22-30 compared to earlier stages. The transcript level of PPARβ was significantly lower on days 11-12 and 22-30 than on days 5-8, 9-10, 13-15. mRNA expression of PPARα was high on days beginning from 5-8 until 18-21 and significantly dropped on days 22-30. The results indicate that the endometrial expression of PPARs genes fluctuates during the estrous cycle and pregnancy. PPARα and PPARβ transcript levels show similar profiles during the estrous cycle. The decrease of both transcripts concentration on days 10-12 and 22-30 days in pregnant gilts implicates their role in maternal recognition of pregnancy and the end of implantation, respectively.

Key words: *implantation, peroxisome proliferator activated receptors α, β, γ, reproduction, estrous cycle, pregnancy*

INTRODUCTION

Peroxisome proliferator activated receptors (PPARs) are ligand-activated receptors belonging to the family of the nuclear hormone receptors. Three members of the PPARs family have been described as -α, -β -γ1 and -γ2. They share a common structure with other steroid hormone receptors and they are encoded by separate genes. PPARs can be activated by natural ligands such as polyunsaturated fatty acids or prostaglandin metabolites (1, 2). They can also be activated by synthetic pharmacological agents, *e.g.* thiazolidinediones and fibrates, which can be used to normalize blood lipids profile and metabolic syndrome in patients with type 2 diabetes (3). For a complete activation, after binding with the ligand, the receptor must heterodimerize with the retinoic acid receptor (RXR) and as a transcription factor it regulates a target gene by interaction with the specific PPAR response element (PPRE) located in a promoter region (4, 5).

PPARs exhibit different pattern of expression, bind diverse ligands and play distinct functions (6, 7). The highest PPARα expression is noted in liver, kidney, heart, and brown fat where it plays a key role in fatty acid catabolism (8). PPARγ1 and PPARγ2 are mainly localized in white and brown adipose tissue where they regulate adipocytes differentiation, glucose and lipid metabolism, mitochondrial biogenesis and inflammatory response (9-14). PPARγ has been also suggested as a differentiation factor and a potential anti-oncogenic target in breast and colon cancer (15). PPARβ is ubiquitously expressed and its role is not well known (16).

Recently PPARs are considered important players in the field of reproduction (17-22). Three isoforms are expressed in the central nervous system and in several reproductive tissues: gonads (ovary, testis), uterus, gestational tissues (placenta, amnion, choriodecidua), prostate, mammary and pituitary gland (23-25). PPARα deletion does not affect fertility in rodents (26), however PPARγ deficiency results in death by the 10th day of pregnancy (9). It has been noted that PPARγ or PPARβ inactivation leads to impaired embryo implantation, placental development and death of the embryos (9, 27-29). Administration of PPARs agonists to pregnant rodents reduces mortality of the fetuses (20).

Several evidences underline the important role of PPARs in the ovary. PPARγ expression has been found in the granulosa, theca and luteal cells (18, 24, 30-32). It has been suggested that

PPARγ regulates the differentiation and proliferation of ovarian cells, steroidogenesis, angiogenesis and prostaglandin production (33-37).

The above observations indicate that PPARs modulate the estrous cycle and pregnancy however their physiological effects were mainly studied at ovary and/or placenta levels in rodents. There is little evidence that PPARs are expressed in other reproductive tissues depending on the physiological status of the animals. Therefore the present study was undertaken to determine the quantitative expression of several isoforms of PPARs mRNA in the porcine endometrium during the estrous cycle and periimplantation period.

MATERIALS AND METHODS

Animals

All procedures were approved by the Local Animal Ethics Committee and study was conducted in accordance with the national guidelines for agricultural animal care.

The studies were performed on crossbred sows of Polish Landrace and Pietrain from a private farm. Animals were kept in the boxes with free access to water and food and maintained under ambient photoperiod and temperature (18-20°C). Gilts with an average body weight of 100 kg and 7 months of age were synchronized and superovulated by single intramuscular injection of 750 I.U. PMSG (Folligon, Intervet) followed by 500 I.U. hCG (Chorulon, Intervet) given 72 h later, as described previously by Kaminska *et al*. (38). Experimental animals were divided into two groups, cyclic and pregnant individuals. Gilts from the first group (cyclic) were slaughtered at the proper day of the estrous cycle to create following subgroups: 2-4 (n=3), 5-8 (n=6), 9-10 (n=3), 11-12 (n=3), 13-15 (n=6), 16-17 (n=3) and 18-21 (n=6).

Gilts from the second group (pregnant) were inseminated twice after treatment with PMSG and hCG (the first time 24 h after hCG treatment and the second time 12 h later) and were slaughtered at a proper day of pregnancy to create following subgroups: 5-8 (n=6), 9-10 (n=6), 11-12 (n=7), 13-15 (n=7), 16-17 (n=5), 18-21 (n=3), 22-30 (n=3). The days of the pregnancy were based on the presence of embryos in uterus horns and the morphology of embryos and corresponded to the days of the estrous cycle except 2-4 and 22-30 stages.

Immediately after slaughter the endometrial tissue was dissected from myometrium, frozen in liquid nitrogen and stored at -70°C until mRNA expression analyses.

RNA extraction and real time PCR

Total RNA from each tissue sample was isolated using total RNA kit (A&A Biotechnology, Poland) according to the manufacture's instructions. RNA samples were quantified spectrophotometrically (Nanodrop, USA) and the integrity was confirmed using 1.5% agarose gel.

Complementary DNA (cDNA) was synthesized by QuantiTect® (Qiagen, USA) as described previously (38).

Real time PCR reactions were performed using TaqMan Gene Expression MasterMix (Applied Biosystems, USA). The sequences of primers and Taqman probes (labeled with 6-FAM) for PPARα, PPARβ, PPARγ1/2 and housekeeping gene - GAPDH (glyceraldehyde-3-phosphate dehydrogenase) - were designed using Primer Express Software 3 (Applied Biosystems, USA). The sequences, access numbers in GenBank and lengths of the products are presented in *Table 1*. Real-time PCR was carried out in duplicates for each sample in the 7300 real-time PCR system (Applied Biosystems, USA) using the following parameters: one cycle of an initial denaturation (10 min at 95°C), followed by 40 cycles of denaturation at 95°C for 15 sec; annealing at 59°C for 1 min and extension at 95°C for 15 sec. The standard curves were prepared for tested and housekeeping genes. In each run the non-template control (NTC) samples were included. All gene expression data were normalized by dividing the mRNA amount of the target gene by the amount of GAPDH mRNA and presented as arbitrary units.

Statistical analysis

All results are presented in figures as mean ±S.E.M. Statistical analyses were performed using Statistica (version 6, StatSoft Inc, Tulsa, USA). Significant differences within cyclic and pregnant groups were established by one-way Anova with least significant differences (LSD) *post hoc* test and assumed as statistically significant for $P<0.05$. To establish the impact of

Table 1. Oligonucleotide primers and probes for real time PCR.

Gene name	GeneBank Accession No.	Primer/Probe sequences	Product lenght (bp)
PPARα	NM_001044526	F: TTTGTGGCTGCTATCATTTGGT R: CCTCCTGCATTCTCTCAATGTG P: CGGCCCGGCCTCCTAAACGTAG	76 bp
PPARβ	NM_214152	F: GCGCCTACCTGAAAAACTTCA R: GCCTTGCCGGTGAGGAT P: CATGACCAAAAAGAAGGCCCGCG	64 bp
PPARγ1	AJ006756	F: CACTAACATACAGGAAGGTTGTTTCCT R: GTCCACAGAGCTGATCCCAAA P: AGATGCCGTTTTGGCCCACCAAC	116 bp
GAPDH	U48832	F: CATCAATGGAAAGGCCATCAC R: CAGCATCGCCCCATTTG P: CTTCCAGGAGCGAGATCCCGCC	68 bp
PPARγ2	AF103946	F: CGATGCCTTCGACACGCT R: GTCCACAGAGCTGATCCCAAA P: AGATGCCGTTTTGGCCCACCAAC	103 bp
PPARγ2	AF103946	F: TGTTATGGGTGAAACTCTGGGAG R: GTCCACAGAGCTGATCCCAAA P: AGATGCCGTTTTGGCCCACCAAC	148 bp
PPARγ2	AF103946	F: CAGAAAGCGATGCCTTCGA R: ACCATGGTCACCTCTTGTGAAA P: ACGCTGTCTGCAAAC	58 bp

stage of the estrous cycle/pregnancy, reproductive status or interaction effect we performed two-way Anova analysis.

RESULTS

Using a quantitative real time PCR we have found the presence of all isoforms (α, β, γ) of the peroxisome proliferator activated receptors (PPAR) mRNA in porcine endometrium during all stages of the estrous cycle and pregnancy in periimplanatation period. We have noted the presence of PPARγ1, while the expression of PPARγ2 was undetectable at a significant level. Three sets of primers/probes were tested and any did not give satisfactory results, although in adipose tissue the expression was detectable. We may suggest that PPARγ1 is a major isoform expressed in the endometrial tissue in pigs. Furthermore, the level of the transcripts fluctuated in the tissue depending on the phase (luteal, follicular) of the estrous cycle and the day of the pregnancy (early and late stages of implantation).

Peroxisome proliferator activated receptor α gene expression

Quantitative expression of PPARα mRNA in the endometrial tissue throughout the estrous cycle and early pregnancy is presented on *Fig. 1A* and *1B*, respectively. During the estrous cycle the markedly low amounts of PPARα transcript were noted on days: 2-4, 16-17 and 18-21 in comparison with the remaining stages (days 5-8, 9-10, 11-12, $P<0.01$). During early pregnancy significantly higher mRNA levels were observed on days: 5-8, 9-10,13-15, 16-17, 18-21 than on days 11-12 and 22-30 ($P<0.05$).

The present study showed significant differences in PPARα mRNA levels between the tested stages of pregnancy in

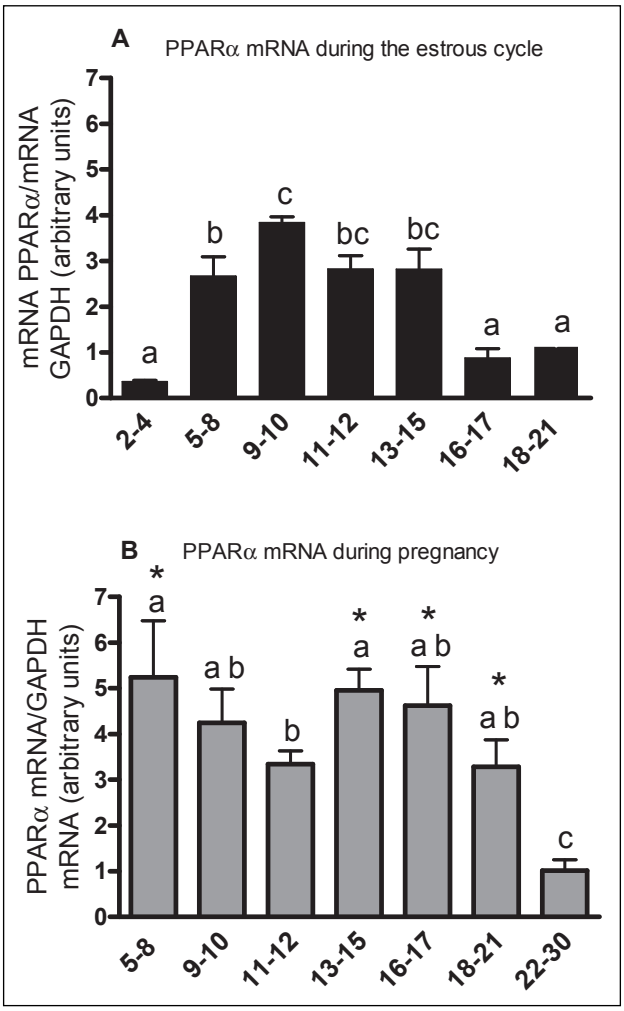

Fig. 1. The expression of peroxisome proliferator activated receptor α (PPARα) gene in porcine endometrium during different days of the estrous cycle (A) and early pregnancy (B). The expression of mRNA has been determined by quantitative real time-PCR. All expression data were normalized by dividing the amount of target gene by the amount of housekeeping gene, GAPDH, and presented as arbitrary units. Differences in transcript levels within experimental group were assumed as statistically significant for $P<0.05$ and marked with different letters. Statistically significant ($P<0.05$) differences in transcript levels between experimental group (stage of the estrous cycle *vs*. corresponding stage of pregnancy) were marked with asterisks.

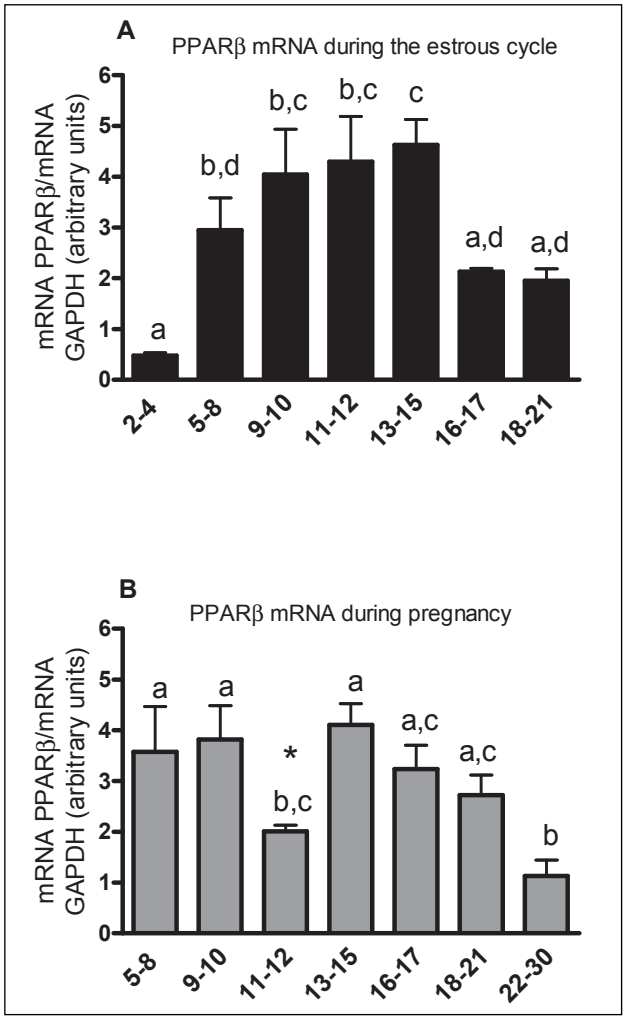

Fig. 2. The expression of peroxisome proliferator activated receptor β (PPARβ) gene in porcine endometrium during different days of the estrous cycle (A) and early pregnancy (B). The expression of mRNA has been determined by quantitative real time-PCR. All expression data were normalized by dividing the amount of target gene by the amount of housekeeping gene, GAPDH, and presented as arbitrary units. Differences in transcript levels within experimental group were assumed as statistically significant for $P<0.05$ and marked with different letters. Statistically significant ($P<0.05$) differences in transcript levels between experimental group (stage of the estrous cycle *vs*. corresponding stage of pregnancy) were marked with asterisks.

comparison with the corresponding days of the estrous cycle. In details, a significantly higher gene expression was observed in the endometrial tissue of pregnant gilts on days: 5-8 (5.24 *vs*. 2.67 arbitrary units, P<0.01), 13-15 (4.95 *vs*. 3.17, P<0.05), 16-17 (4.62 *vs.* 0.88, P<0.01), 18-21 (3.29 *vs*. 1.14, P<0.05) than on the corresponding days of the estrous cycle. In the remaining stages (9-10 and 11-12) of gestation and the estrous cycle there was no difference in the transcript levels.

We noted a significant impact of the estrous cycle/pregnancy days (F=3.256; p=0.090) and the reproductive status (F=27.436; p=0.000001) but not the interaction between these two parameters (F=1.952; p=0.091) on the PPAR mRNA expression.

Peroxisome proliferator activated receptor β gene expression

The expression of PPARβ mRNA in the endometrial tissue throughout the estrous cycle and pregnancy is presented on *Fig. 2A* and *2B*, respectively. During the estrous cycle the diminished levels of PPARβ mRNA were observed on days 2-4, 16-17 and 18-21 in comparison with the remaining stages (days 5-8, 9-10, 11-12, P<0.01). During pregnancy the transcript level of PPARβ was significantly lower (p<0.05) on days 11-12 and 22-30 than on days 5-8, 9-10, 13-15.

When compared mRNA levels of PPARβ during the cycle to the corresponding days of pregnancy we noted significantly higher expression at stage 11-12 of the estrous cycle (4.30 *vs*. 2.01 arbitrary units, P<0.05) than at corresponding stage of the pregnancy.

We noted a significant impact of the estrous cycle/pregnancy days (F=3.55; p=0.005), but not the reproductive status (F=0.064; p=0.801) or the interaction between these two parameters (F=1.992; p=0.084) on the PPARβ mRNA expression.

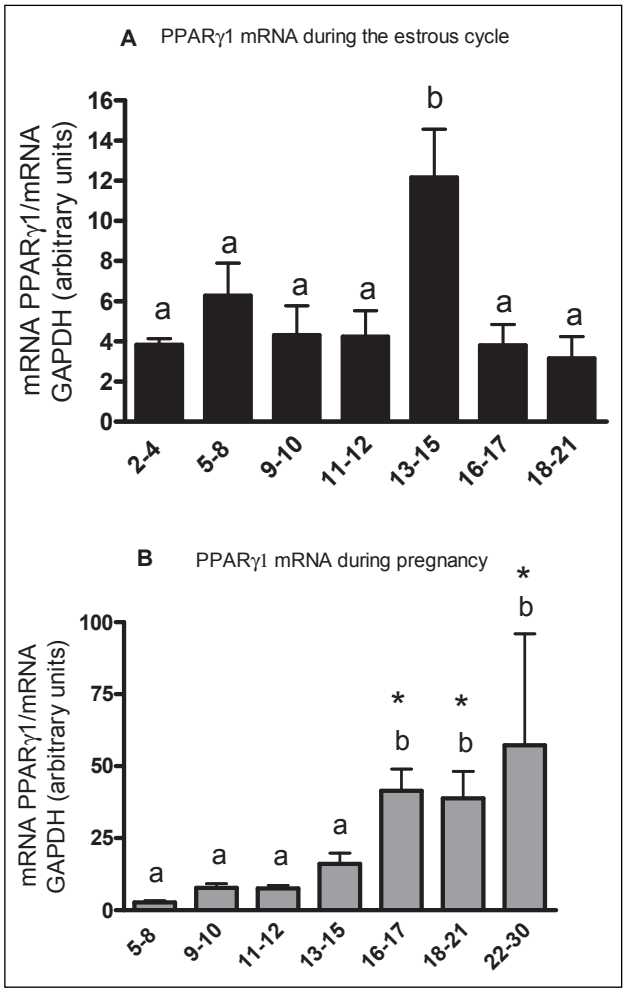

Fig. 3. The expression of peroxisome proliferator activated receptor γ1 (PPARγ1) gene in porcine endometrium during different days of the estrous cycle (A) and early pregnancy (B). The expression of mRNA has been determined by quantitative real time-PCR. All expression data were normalized by dividing the amount of target gene by the amount of housekeeping gene, GAPDH, and presented as arbitrary units. Differences in transcript levels within experimental group were assumed as statistically significant for P<0.05 and marked with different letters. Statistically significant (P<0.05) differences in transcript levels between experimental group (stage of the estrous cycle *vs*. corresponding stage of pregnancy) were marked with asterisks.

Peroxisome proliferator activated receptor γ1 gene expression

The expression of PPARγ1 mRNA in the endometrial tissue throughout the estrous cycle and pregnancy is presented on *Fig. 3A* and *3B*, respectively. During the estrous cycle PPARγ1 mRNA level was significantly higher (p<0.01) on days 13-15 than on the remaining days. During pregnancy significant increase (p<0.01) in the mRNA expression was noted on days 16-17, 18-21 and 22-30 in comparison with earlier stages.

Additionally, when compared PPARγ1 mRNA levels between the corresponding stages of the estrous cycle and pregnancy we observed significantly higher PPARγ1 gene expression (P<0.001) on days 16-17, 18-21 of gestation than on the corresponding days of the estrous cycle (40.00 *vs*. 3.80; P<0.00001 and 38.87 *vs*. 3.16, P<0.00001, respectively).

We noted a significant impact of the estrous cycle/pregnancy days (F=5.263; p=0.0005), the reproductive status (F=25.403; p=0.000005) and the interaction between these two parameters (F=6.819; p=0.00005) on the PPARγ1 mRNA expression.

DISCUSSION

In the present study we have demonstrated that three isoforms of peroxisome proliferator activated receptors (α, β, γ1) mRNAs are expressed in the porcine endometrial tissue during the estrous cycle and early pregnancy. We have also noted that their gene expression fluctuates depending on the phase of the estrous cycle or the stage of pregnancy.

In details, during the estrous cycle we observed low PPARα and PPARβ transcript levels on the first days of the luteal phase (stages 2-4). After that, mRNA levels markedly raised and maintained high until 13-15 days. Starting from days 16-17 until the end of the estrous cycle, both PPARs mRNA levels significantly dropped and did not differ from that observed at the beginning of the luteal phase. PPARγ1 mRNA level was quite stable during entire estrous cycle. Only on days 13-15 (functional luteolysis) its mRNA expression rapidly increased.

A different pattern in the gene expressions data was observed during early pregnancy. We noted a stable expression of PPARα and β on days 5-21 of gestation. However on days 11-12 (maternal recognition of pregnancy) and 22-30 (the end of implantation) mRNA levels of both receptors were markedly reduced. PPARγ1 gene expression was stable and low at the beginning of gestation (days 5-15) and after that significantly increased and maintained high until day 30.

The role of PPARs in the uterus is not well known although PPARα, β or γ expression have been documented in human, bovine, porcine and rodent uterus (39-45). There is also study showing the lack of PPARγ gene expression in bovine endometrial cell line (BEND) (41). Based on other reproductive

tissues we may suggest a possible interaction of PPARα/β mRNA expression with steroids in the endometrium and no such clear correlation of PPAγ1.

There is few evidence documenting possible interactions between PPARγ and steroids (estrogen) signaling in uterus. Keller *et al*. (46) for the first time reported a possible signaling cross-talk between PPAR/RXR and estrogen receptor. PPARγ activation by rosiglitazone enhanced proliferative estrogen action and evoked estrogen-dependent endometrial hyperplasia in mouse uterus (47). In the same study it has been shown that PPARα activation by fenofibrate had opposite effect and attenuated proliferative action of estrogens (47). In another study the activation of PPARγ inhibited growth of estrogen-dependent uterine leiomyoma (40) and this effect was probably mediated by inhibitory action of PPARγ on estrogen receptor signaling (40). In another experimental model it has been shown that treatment with estradiol inhibited activation of PPARγ in breast cancer cells (48).

Based on our study we may propose a correlation between PPAR gene expression and steroids. The lower expression of PPARα/β noted at the early beginning of luteal phase and later during follicular phase of the estrous cycle, when progesterone level is diminished, but when estrogens play a crucial role, may suggest such association. This observation can also be supported by the observation that their mRNA levels significantly drop on days 11-12 of gestation when estrogens, produced by the embryos, play an important role during maternal recognition of pregnancy in pigs. We can also observe a gradual decrease of mRNA levels of the two PPARs gene expression, starting from days 18-21 until day 30. It can be also a result of a higher local estrogen level.

Lord *et al*. (44) reported the expression of PPARβ and PPARγ in porcine endometrium on day 15 of the estrous cycle and day 15 and 25 of pregnancy. They noted a higher gene expression of PPARβ on day 15 of the estrous cycle compared to the two tested stages of pregnancy. However, a lower PPARγ1 transcript level was described on day 15 of the estrous cycle than on day 25 of pregnancy and additionally a higher level on day 25 than on 15 (44). In our experiment we quite similarly observed higher expression of PPARγ1 mRNA on days 16-30 of pregnancy than on days 5-15 and a lower expression on days 16-17 and 18-21 of the estrous cycle in comparison with the corresponding days of gestation. In case of PPARβ we have shown a higher gene expression only at stage 11-12 of the estrous cycle than on the corresponding days of pregnancy. The expression of PPARα was markedly higher in endometrial tissue of pregnant animals on days 5-8, 13-15, 16-17, 18-21 than on the corresponding days of the estrous cycle.

There are limiting data describing the PPARs expression in endometrial tissue of other animal species through the estrous cycle or pregnancy. The expression profile of PPARs during pregnancy has been recently reported by Nishimura *et al*. (55). They demonstrated that PPARγ gene expression in rat uterus significantly decreased at 2.5 dpc (days post-coitum) and remained low until day 6.5 (during implantation). PPARα and PPARβ gene expression showed different pattern. Although no significant changes in mRNA level was observed at 0.5-6.5 dpc, immunostainnig results showed differences in the endometrial stroma on days 4.5-6.5 of pregnancy. In previously published data it has been reported that in mice, a very low level of PPARβ expression in the subluminal stroma surrounding the implanting blastocyst in mouse uterus has been reported on days 1-4 during gestation and a rapid increase was observed from day 5, during implantation (43). In rats, PPARβ mRNA expression in the luminal epithelium was high on day 1 of pregnancy, then declined and was undetectable until day 5 (42). However, the expression in the glandular epithelium increased from day 2 and was highest on day 5 of pregnancy (42).

There is a number of ways where PPARs could regulate physiological function of the uterus. The endometrium is a possible place where PPARs may regulate cyclooxygenase (COX)-2 (COX-2) activity, the rate-limiting enzyme in prostaglandins production. They are critical to sustain the corpus luteum function during the estrous cycle or pregnancy. The PPAR response element has been found upstream of the COX-2 transcriptional start site (49) and the activation of PPARs affects COX-2 gene/protein expression in mammary epithelial cells or corneal epithelium (49-51). Additionally, the treatment of bovine endometrial cells with PPAR agonists affects prostaglandins ($F_2\alpha$, E_2) *in vitro* accumulation (41). Interestingly, in COX-deficient mice failures during the implantation and decidulaization may be restored by administration of PPARβ agonists suggesting common pathways of both agents (52-54).

In the present study we observed a marked increase in PPARγ1 mRNA expression on days 13-15 of the estrous cycle (during luteolysis) and the decrease in PPARα/β at stage 11-12 of gestation (maternal recognition of pregnancy). Above observations suggest an involvement of the receptors in the production/secretion of uterine prostaglandins. In our next study, we plan to explore the PPARs and prostaglandins associations.

Moreover, the comparison with other cell models (*e.g.* ovarian cells) suggests that PPARs participate in uterine physiological functions such steroidogenesis, cytokines production, tissue remodeling or angiogenesis during the estrous cycle and pregnancy (18). However this also needs to be verified in the future experiments.

CONCLUSIONS

The expression of PPARs genes in the porcine endometrium changes throughout the estrous cycle and early pregnancy. PPARα and PPARβ transcript levels show similar profiles during the estrous cycle. High levels during the luteal phase and low during the follicular imply their association with steroids. The decrease of both transcripts concentration on days 10-12 and 22-30 in pregnant gilts implicates their role in maternal recognition of gestation and the end of implantation, respectively. High PPARγ1 mRNA level on days 13-15 of the estrous cycle suggest its role during luteolysis.

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Conflict of interests: None declared.

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