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Original article

The novel effect of hCG administration on luteal function maintenance during the estrous cycle/pregnancy and early embryo development in the pig

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Abstract

Two independent experiments were performed on cyclic (Experiment I) and pregnant (Experiment II) gilts to examine the effect of human Chorionic Gonadotropin (hCG) administration on day 12 of the estrous cycle/pregnancy on ovarian and endometrial secretory function. Animals were divided into hCG Group (injection of 750 IU hCG) and Control Group (injection of saline). In Experiment I, the prolonged lifespan of the corpus luteum (CL), extended progesterone (P4) production ($P<0.05$) and delayed luteolysis were found. In hCG Group increased ratio of PGE2:PGFM during 12 hrs period on day 15 ($P<0.05$) of the estrous cycle was observed. In both experiments, higher concentrations of E2 in hCG treated gilts ($P<0.05$) on days 14-15 of the estrous cycle/pregnancy were found. In Experiment II, hCG injection did not affect P4, PGE2 and PGFM concentrations in blood plasma, but reduced the number of resorbed embryos on day 30 of pregnancy. In the pregnant hCG treated gilts the immunostaining against von Willebrand Factor (vWF) demonstrated an enhanced ($P<0.05$) angiogenesis in CLs and endometrium. Furthermore, the flow cytometry revealed an increased ($P<0.05$) viability of cells in CLs of hCG Group. An augmented expression of Steroidogenic Acute Regulatory Protein (STAR; $P<0.05$) and LH/hCG receptor mRNA ($P<0.05$) in CLs of hCG Group were observed, but an elevated concentration of protein was confirmed only for STAR ($P<0.05$). Our studies revealed, for the first time, that administration of hCG affects PGE2:PGFM ratio during the estrous cycle as well as the development of conceptuses through enhanced angiogenesis and decreased luteal apoptosis in early pregnant pigs.

Key words: hCG, corpus luteum, early embryonic mortality, insufficient luteal function

Introduction

A concentration of progesterone (P4) is positively correlated with embryonic survival during the first week of gestation (Jindal et al. 1997). The majority of embryonic losses (20-30%) in pigs occurs between days 12-30 of gestation (Lambert et al. 1991). Further embryonic mortality takes place during the mid- to late gestation and accounts 10-15% (Vallet et al. 2011).

Despite many endeavours, P4 supplementation during the early pregnancy yielded an inconsistent results. Some studies showed an improved embryonic rates (Jindal et al. 1997), whereas the others indicated its negative effect on fertilization rates (Day and Polge 1968) or embryo survival rates (Mao and Foxcroft 1998). Unsuccessful attempts proved also treatments with estradiol (E2; Vallet and Christenson 2004) considered as a main luteotrophic and antyluteolytic factor in the pig (see: Ziecik et al. 2002) and responsible for increased uterine blood supply (Ford et al. 1982). On the other hand, the premature time exposure (days 9-10 post-insemination) of the uterus to E2 altered the expression of some endometrial genes and led to pregnancy failure (Geisert et al. 2006).

Some data highlighted the beneficial effect of human Chorionic Gonadotropin (hCG) on P4 concentration and embryonic survival in the cow (Rajamahendran and Sianangama 1992) and sheep (Khan et al. 2007). The reduced embryonic mortality after hCG administration was also observed in pigs (Tilton et al. 1989), however, without an increased concentration of P4 and the possible mechanism of hCG action on improved embryonic rate in pigs remains unknown. The luteal function maintenance during the estrous cycle and decreased embryonic losses in pigs could result from an elevated amounts of estrogens after hCG administration (Guthrie and Bolt 1983, Tilton et al. 1989). Guthrie and Rexorad (1981) also demonstrated a two-fold increase of E2 in the peripheral circulation following hCG injection in cyclic gilts.

The luteinizing hormone (LH) participates in prostaglandins (PGs) production (Stepien et al. 1999) and releasing in the pig (Ziecik et al. 2001, Blitek and Ziecik 2005). PGs play an essential role in the luteal function maintenance (Gregoraszczyk and Michas 1999) and implantation (Waclawik et al. 2006). Thus, LH/hCG may also indirectly affect the luteal function during the estrous cycle and early pregnancy as well as the embryo development in pigs. Considering an aforementioned roles of PGs and the novel angiogenic role of LH/hCG (Zygmunt et al. 2002) in female reproduction we decided to extend studies on

the effect of hCG on the luteal function during the estrous cycle and pregnancy in the pig.

Consequently, the aim of this study was to evaluate the effect of hCG administration on day 12 of the estrous cycle/pregnancy on ovarian and endometrial secretory (luteolytic/luteotrophic activity) function. Additionally, the mechanism underlying the action of hCG on steroidogenesis, apoptosis and angiogenesis in luteal tissue and inter-implantation sites of the endometrium during the period of early pregnancy in pigs was also analysed.

Materials and Methods

Animals and samples collections

All procedures performed on animals were conducted in accordance with the rules approved by Local Animal Ethics Committee. Crossbred gilts 6-8 months of age after one regular estrous cycle were assigned to the experiment performed at the commercial farm. The animals were kept in individual pens and observed daily for the estrus behavior. The first day of the estrus was designated as day 0 and gilts were randomly assigned into followed Experiments described below.

Ten crossbred cyclic gilts during their second estrous cycle were randomly divided in two groups (n=5 per group): hCG Group- given intramuscular (i.m.) injection of 750 IU of hCG (Chorulon, Intervet, Warsaw, Poland) and Control Group- given i.m. injection of saline on day 12 of the estrous cycle (Experiment I).

Fourteen crossbred (n=7 per group) pigs were artificially inseminated 24 and 48 hrs after estrus detection and randomly divided in two groups: hCG Group (i.m. injection of 750 IU of hCG) and Control Group (i.m. injection of saline) on day 12 post-insemination. The pregnancy was confirmed on day 22 using ultrasonography. All inseminated animals were considered as pregnant and kept in pens until day 30 of pregnancy and then slaughtered.

A catheter was inserted into the jugular vein under general anaesthesia to five (cyclic) and four (pregnant) gilts on day 9 of the estrous cycle or pregnancy, according to the method of Kotwica et al. (1978). Blood samples (~ 5 ml) were collected twice per day (at 12 h intervals) on days 11-19 of the estrous cycle and pregnancy and then centrifuged for 15 min at 3000 rpm at 4°C. To determine the frequency of peaks of PGF2 α metabolite- 13,14-dihydro-15-keto-PGF2 α -1 (PGFM) and PGE2, blood samples every 2 h over 12 hrs period on day 13 and 15 of the estrous cycle were collected. The plasma samples were stored at -20°C until assayed for hormones concentrations.

The animals were slaughtered at a local abattoir on day 20 of the estrous cycle or day 30 of pregnancy. In all cyclic and pregnant gilts the assessment of uterine and ovarian morphology was performed. Additionally, in pregnant pigs each uterine horn was cut longitudinally on the antimesometrial surface. After dissection of embryos from the surrounding fetal membranes, the total number of embryos and number of resorbed embryos were calculated. Furthermore, the part of corpora lutea (CLs) and inter-implantation site of endometrial tissue samples from pregnant pigs were snap frozen in liquid nitrogen and stored at -80°C for further analysis. For immunohistochemical staining, tissue samples were fixed in 4% paraformaldehyde in 0.1M Phosphate-Buffered Solution (PBS; pH 7.4). The luteal tissue was placed in PBS supplemented with antibiotics (100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin) and transported on ice to the laboratory to examine the apoptosis.

Determination of hormones concentrations

Determinations of PGE₂ and PGFM concentration in blood plasma were performed using EIA method (Blitek et al. 2010). Anti-PGE₂ antibodies (P-5164; Sigma-Aldrich) were used at dilution 1:450. Assay sensitivity was 0.19 ng/ml. Intra- and interassay coefficients of variation were 8.5% and 8.1%, respectively. Anti-PGFM serum (WS4468-7; donated by Dr. Seiji Ito, Kansai Medical University, Osaka, Japan) was used at a dilution of 1:80,000. Assay sensitivity was 12.5 pg/ml and the intra- and interassay coefficients of variation were 10% and 11.5%, respectively.

Concentrations of E₂ (Orion Diagnostica, Finland) and P₄ (DIAsource ImmunoAssays S.S, Belgium) were analyzed using RIA commercial available kits. The sensitivity of E₂ assay was 3.12 pg/ml and intraassay coefficient of variation was 6.5%. The sensitivity of P₄ assay was 0.19 pg/ml, whereas intraassay coefficient of variation was 4.6%.

Disaggregation of corpus luteum cells and flow cytometry analysis

The CLs were dissected from the stroma of the ovary of each pig, pooled and gently minced with a sterile scalpel blade. Then, placed in medium M-199 (Sigma-Aldrich) supplemented with 0.1% BSA (ICN Biomedicals, Inc., Costa Mesa, CA; w/v), antibiotics (100 IU/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin), 0.6 U/ml dispase and 0.5 mg/ml DNase type I (Invitrogen Life Technologies Inc., Carlsbad, CA, USA). The low amounts of dispase were chosen to avoid an elevation

of apoptotic-/necrotic like changes in cells. An eventual risk of aforementioned effect occurrence was eliminated by the fact that in all groups the procedure of disaggregation was the same. Three incubations in a shaking water bath at 37°C for 15 min were performed. The contents of the flasks were carefully drawn out, filtered (Biosciences, Bedford, USA) and centrifuged at $200\times g$ for 10 min. Then, washed twice with medium M-199 (Sigma-Aldrich) without enzymes supplement.

The apoptosis of CL cells was determined using FACS Aria II (BD Biosciences) and Dead Cell Apoptosis Kit with Annexin V-FITC and PI (Molecular Probes Life Technologies Inc.) according to manufacturer's protocol. Four populations of cells were distinguished: (1) non-apoptotic cells; (2) early apoptotic cells; (3) late apoptotic cells; (4) necrotic cells.

Immunostaining for von Willebrand Factor

The immunostaining against von Willebrand Factor (vWF) was performed as described previously by Kaczmarek et al. (2010). Polyclonal rabbit anti-human vWF antibodies (marker of endothelial cells; 1:500; DakoCytomation) were detected with goat anti-rabbit IgG biotin conjugated secondary antibody (1:5000; Vector Laboratories Inc.). The specificity of antibodies was confirmed using following type of controls: (1) the primary antibodies were omitted during the immunostaining procedure, and (2) were replaced with normal rabbit serum diluted in the same protein concentration as the primary antibody (NRS, DakoCytomation). The sections were analysed under a light microscope (Olympus BX 60; Olympus Optical Co. Ltd., Tokyo, Japan) and photographed using an Olympus digital camera.

To calculate the density of blood vessels regions from the inner, central and outer area of the luteal sections and from the outer area (under the epithelium) of the endometrium were randomly selected and examined at $\times 400$ magnification. Each positive stained signal was counted. Number of capillaries for the individual animal was expressed as mean from each tissue section per area unit (microscopic field- $5.7 \times 10 \mu\text{m}^2$).

Gene expression analysis

Isolation of RNA, Reverse Transcription and Real-Time PCR were performed according to the method described by Blitek et al. (2010).

Primers for genes were used according to the literature or designed by Primer3 software and using

Table 1. Investigated genes, accession numbers/references, primer sequences and product length.

Gene name	Primers sequence 3' → 5' #	Product size (bp)	Gene Bank Accession Number/References
LHCGR	F:CGAAAGCTTCCAGATGTTACGAA R:CATTGAAGGCATGACTTTGTATTCT	181	NM_214449.1
STAR	F:CTCTGGCTGGAAGTCCCTCAA R:CAAAGTCCACCTGGGTCTGTGA	117	NM_213755.2
HSD3B	F:TCCACACTGCCTCTATCATCGAC R:CGTTCTGGATGACCTCCCTGTA	183	NM_001004049.1
VEGF164	F:GAGGCAAGAAAATCCCTGTG R:TCACATCTGCAAGTACGTTTCG	150	NM_214084 Kaczmarek et al. (2008)
VEGF120	F:AAGGCCAGCACATAGGAGAG R:CCTCGGCTTGTCACATTTTT	101	NM_214084 Kaczmarek et al. (2008)
BCL-2	F:CGCCCTGTGGATGACTGAGTA R:CCCGTGGACTTCACTTATGGC	212	NM_214285.1
BAX	F:ACATCAAGGAGAAGCTCTGCTACG R:GAGGGGCGATGATCTTGATCTTCA	238	AJ606301 Feugang et al. (2011)
ACTB	F:CCTTCATTGACCTCCACTACATGGT R:CCACAACATACGTAGCACCACGATC	366	U07786 Kaczmarek et al. (2010)
GAPDH	F:TTTCTGACGGCAACTTCAACTG R:AGCCACAAAGATGGTCACTGTCT	183	U48832 Kaczmarek et al. (2010)
PPIB	F:TTG TGG CCT TGG CTA CAG GA R:GTT CTC GTC GGG AAA GCG TT	155	XM_001927047.2

F, forward; R, reverse.

cDNA sequences from Gene-Bank (Table. 1). The temperature of primers annealing for all genes was 60°C. Specificity of reaction was confirmed using melting curve after each PCR reaction. Additionally, agarose gel electrophoresis and sequencing for new designed primers were performed. Relative amounts of investigated mRNA and housekeeping gene were determined from the standard curve. The most stable housekeeping genes (for the luteal tissue-PPIB; for the endometrial tissue-ACTB) were chosen by NormFinder algorithm.

Protein expression analysis

The luteal tissue homogenization and Western Blot analysis were performed as described previously (Kaczmarek et al. 2007) with some modifications. The equal amounts of cytosol (for STAR-20 µg) and microsomal (for LH receptor-10 µg) fractions were separated on 10% SDS-PAGE and transferred onto nitrocellulose/PVDF membranes. Primary antibodies were used as follows: polyclonal rabbit anti-STAR (1:500; Abcam, Cambridge, UK), polyclonal rabbit anti-LHR (1:500; donated by Dr Patrick C.Roche, Mayo Clinic, Rochester, MN, USA) and polyclonal anti-β-actin (ACTB; 1:3000; Abcam, Cambridge,

UK). The secondary antibodies were anti-rabbit alkaline phosphatase-conjugated (1:20,000; Sigma-Aldrich) or anti-rabbit horseradish peroxidase-conjugated (1:20,000; BioRad). ACTB was used as an internal control.

Statistical analysis

The concentrations of hormones are expressed as a mean from the two blood sample collections on each experimental day. To analyse differences in the concentration of hormones between examined groups on particular days of the estrous cycle and pregnancy two way ANOVA followed by Bonferroni post *hoc* test was used (GraphPad Prism v. 5.0, GraphPad Software, San Diego, CA, USA). The initial model included day, treatment and treatment by day of estrous cycle/gestation interactions. The data of Control and hCG Groups (no. of CLs, no. of corpora albicantia, weight of ovaries, no. of embryos, P4 concentration on day 30 of gestation, data from apoptosis, genes and proteins expression, no. of capillaries) were compared using an unpaired t student test. In all experiments, P<0.05 was considered to be statistically significant and numerical data are expressed as means ± standard error of mean (SEM).

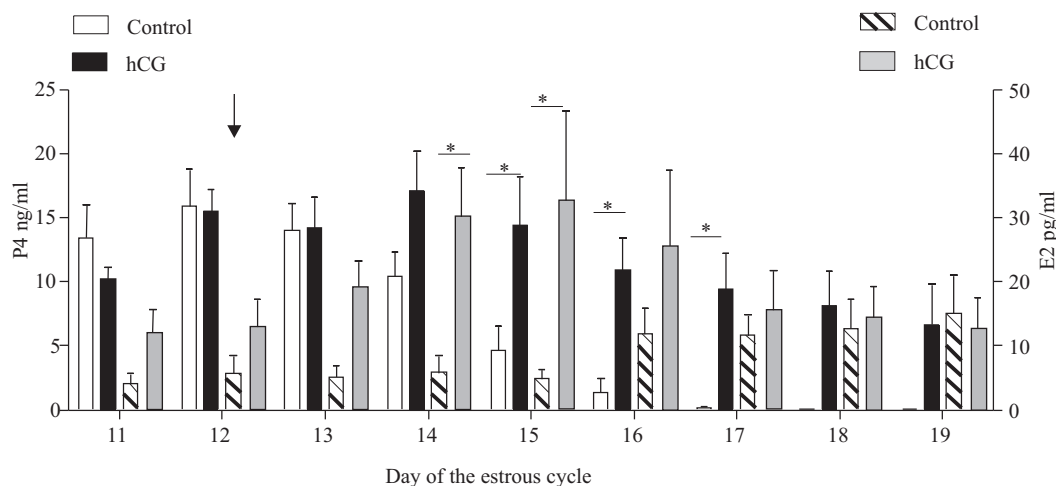


Fig. 1. Mean plasma P4 and E2 concentrations following hCG (hCG Group) and saline (Control Group) administration (Experiment I). Values are expressed as mean \pm SEM (n=5). Bars with asterisks present differences (*P<0.05) between experimental groups on the particular day of the estrous cycle. \downarrow hCG/saline injection.

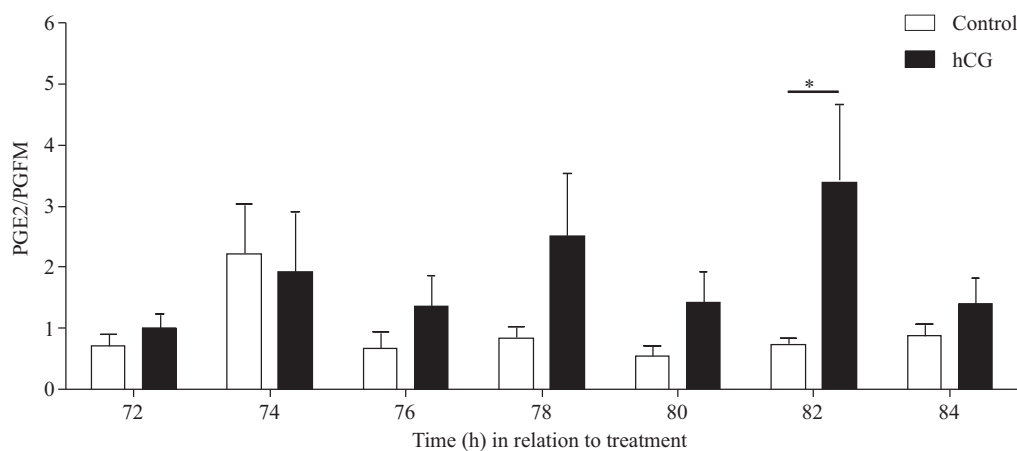


Fig. 2. Ratio of PGE2:PGFM in hCG and Control during 12 hrs period on day 15 of the estrous cycle (Experiment I). Values are expressed as mean \pm SEM (n=5); (*P<0.05).

Results

Experiment I

Administration of hCG prolonged the lifespan of CLs in cyclic gilts when compared to saline given gilts, in which the preovulatory ovarian follicles were observed. The significant differences in the number of corpora albicantia (23.0 ± 3.9 vs 8.7 ± 2.2 for Control Group and hCG Group, respectively) and ovarian weight (5.5 ± 0.5 vs 10.1 ± 1.9 for Control Group and hCG Group, respectively) were found (P<0.05). In one pig of hCG Group two follicular cysts (diameter about 2 cm) were observed.

The concentration of P4 and E2 were affected by day of the estrous cycle (P<0.0001; P<0.05) and day by treatment interaction (P<0.0001; P<0.01). Injection of

hCG increased plasma P4 concentration on days 15-17 in comparison to Control Group (P<0.05; Fig. 1), where content of P4 declined on day 14 of the estrous cycle. In pigs of hCG Group elevated amounts of E2 were observed on days 14 and 15 (P<0.05; Fig. 1). The low stable levels of E2 were observed in Control Group on days 11-15. Then E2 concentration raised gradually from day 16 of the estrous cycle. An overall effect of the treatment on the concentration of PGFM was observed (P<0.05), whereas the content of PGE2 was affected by day of the estrous cycle (P<0.001). There were no statistical differences in the content of PGFM and PGE2 between the groups examined (data not shown). However, in hCG Group the amounts of PGFM started to decline after day 14. In Control Group an elevated amounts of PGFM were observed on days 15-17. The increased ratio

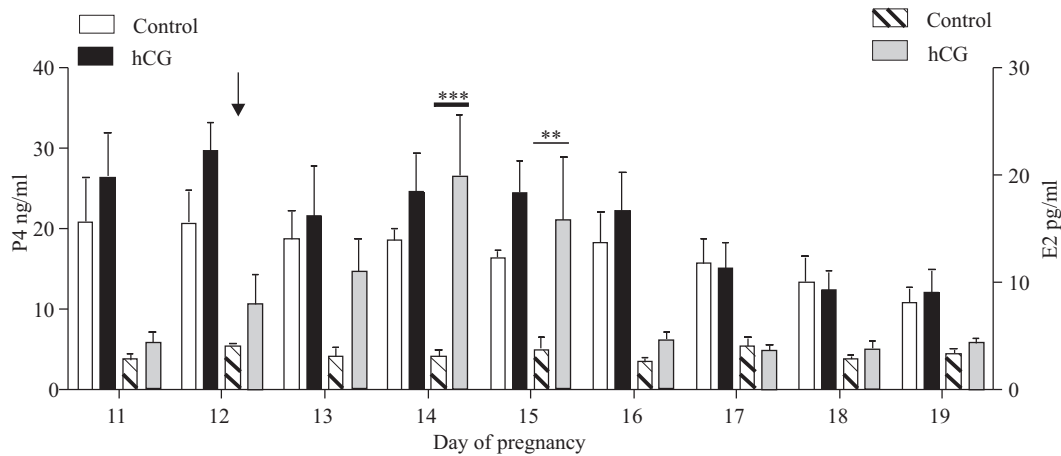


Fig. 3. Mean plasma P4 and E2 concentrations following CG (hCG Group) and saline (Control Group) administration (Experiment II). Values are expressed as mean \pm SEM ($n=4$). Bars with asterisks present differences ($*P<0.05$) between experimental groups on the particular day of pregnancy. \downarrow hCG/saline injection.

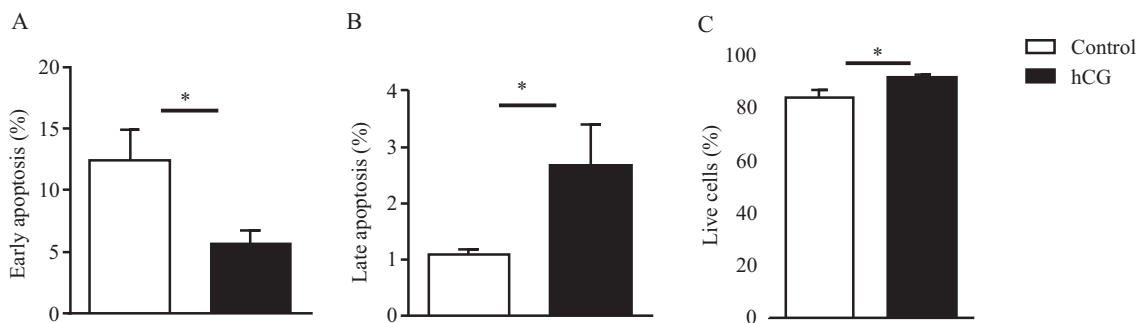


Fig. 4. Analysis of apoptosis in the corpus luteum on day 30 of pregnancy in Control Group and hCG Group ($n=7$ per group; Experiment II). Results obtained are expressed as percentage of: (A) early apoptotic cells; (B) late apoptotic cells; and (C) live cells. Data are expressed as mean \pm SEM; ($*P<0.05$).

of PGE₂:PGFM during 12 hrs period on day 15 of the estrous cycle in hCG Group was determined ($P<0.05$; Fig. 2). No changes in PGE₂:PGFM ratio during 12 hrs period on day 13 were observed.

Experiment II

Macroscopic examination of ovaries showed the difference ($P<0.05$) in the number of CLs between Control Group and hCG Group, averaging 15.3 ± 1.3 and 20.4 ± 1.3 , respectively. The difference in mean number of embryos per group was not statistically significant (13.1 ± 1.5 vs 15.0 ± 0.9 , for Control Group and hCG Group, respectively), but hCG administration reduced the number of resorbed embryos (7.6% vs 0% for Control Group and hCG Group, respectively).

There were no significant differences between examined groups in the concentration of P4 on days 11-19 (Fig. 3) and day 30 of pregnancy (13.1 ± 2.0 vs 15.1 ± 1.7 ng/ml for Control Group and hCG Group,

respectively). An overall effects of day of pregnancy ($P<0.001$), day by treatment interaction ($P<0.001$) and treatment ($P<0.05$) on the concentration of E2 were observed. The level of E2 (Fig. 3) increased after hCG injection on days 14 ($P<0.0001$) and 15 ($P<0.01$). There were no significant differences between examined groups in the content of PGE₂ and PGFM (data not shown).

The reduced percentage of cells in the stage of early and late apoptosis as well as greater percentage of live cells ($P<0.05$; Fig. 4A-C) after hCG administration were observed. No changes were found between the examined groups in percentage of necrotic cells.

The expression of LHCGR and STAR mRNA increased in the luteal tissue of hCG-treated gilts on day 30 of pregnancy when compared to Control Group ($P<0.05$; Fig. 5A-B). Western Blot analysis revealed an increase in STAR protein expression in the luteal tissue of animals from hCG Group ($P<0.05$). No differences in the expression between the rest of studied genes in the luteal tissue were found. Similarly, no differences in the expression of both VEGF isoforms

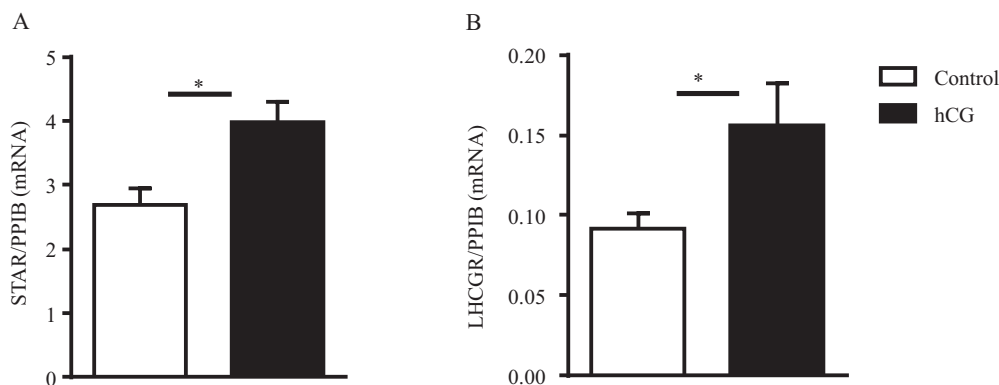


Fig. 5. Expression of STAR (A) and LHCGR (B) mRNA in the luteal tissue on day 30 of pregnancy in Control Group and hCG Group (n=7 per group; Experiment II). All values obtained were normalized to PPIB. Data are expressed as mean \pm SEM; (*P<0.05).

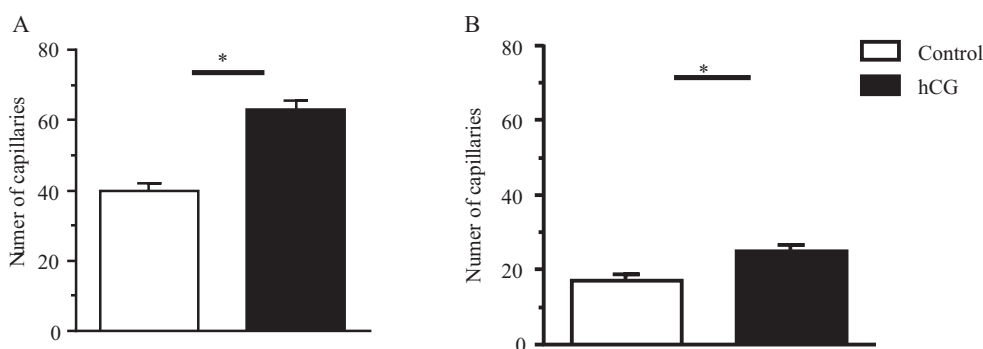


Fig. 6. Number of capillaries in luteal (A) and inter-implantation sites of endometrium (B) expressed per size of examined tissue areas ($5.7 \times 10 \mu\text{m}^2$) and as a mean \pm SEM; *P<0.05 (Experiment II).

in inter-implantation site of the endometrium were determined (data not shown).

The immunostaining for vWF revealed increased (P<0.05; Fig. 6A) angiogenesis in CLs and inter-implantation sites of the endometrium (P<0.05; Fig. 6B) in gilts of hCG Group in comparison to Control Group on day 30 of pregnancy.

Discussion

Human Chorionic Gonadotropin (hCG) has been applied to the estrus synchronization, superovulation and luteal function maintenance due to sharing the same binding site as LH in LH/hCG receptor on granulosa and luteal cells. The beneficial effects of hCG supplementation on P4 production and embryonic mortality (see: Introduction) in domestic ruminants has been highlighted. Therefore, supplementation with hCG seems to be a promising method to reduce the embryo mortality also in the other domestic animals.

The present study confirmed work of Guthrie and Bolt (1983), who reported that single injection of hCG on day 12 of the estrous cycle prolonged the lifespan of CLs, extended synthesis of P4 and delayed luteolysis in pig. In agreement with the above report any significant differences in plasma PGFM concentration between examined groups in our studies were demonstrated. However, the lower levels of PGFM in blood plasma of hCG given gilts after day 14 of the estrous cycle were observed. Administration of hCG had no effect on PGE2 content during the estrous cycle. Nevertheless, it is likely that the reduced concentration of PGFM in hCG given gilts affected the ratio of PGE2:PGFM on day 15 of the estrous cycle. The endometrial PGE2 positive feedback loop and E2 are involved in the mechanism of an increased luteoprotective PGE2:PGFM ratio during the maternal recognition of pregnancy in the pig. Additionally, E2 down-regulates endometrial PGF2 α synthase (PGFS) and prostaglandin 9-ketoreductase (CBR1) protein concentrations leading to an elevated PGE2:PGF2 α ratio (Waclawik et al. 2009). Therefore, the greater

concentration of E2 and increased ratio of PGE2:PGFM could be the main reasons of the prolonged luteal function in cyclic gilts given hCG.

On the contrary to cyclic gilts, administration of hCG had no effect on the plasma P4 concentration of pregnant gilts. Such finding is in accordance with a previous reports, where neither hCG injection during the first 8 days of gestation (Stone et al. 1987) nor on day 12 of pregnancy (Tilton et al. 1989) affected P4 production. This effect may be associated with accelerated metabolism of P4 and its active transport into the uterus rather than with a reduced effect of hCG on luteal function during the gestation. On the other hand, Khan et al. (2007) and Rajamahendran and Siangama (1992) observed elevated amounts of P4 after hCG administration on day 12 of pregnancy in sheep and cows, respectively. However, in these studies the concentration of P4 was positively correlated with an increased number of additional CLs. Interestingly, in our study the significantly higher number of CLs in hCG given gilts were found on day 30 of pregnancy. Nevertheless, this effect probably was not caused by an increase in CLs number since the elevated concentration of P4 was observed before hCG administration. It might be supported by previous report (Tilton et al. 1989), where neither injection of 500 IU nor 1000 IU of hCG had effect on the number of CLs in pregnant gilts.

In the present study, greater amounts of E2 on days 14 and 15 of pregnancy in hCG treated pigs were observed. Similar effects were revealed in pregnant pigs (Tilton et al. 1989) or sheep (Khan et al. 2007). Considering the important role of E2 in a preparation of the uterus to the embryos implantation (Geisert et al. 1990), it is suggested that observed reduction of resorbed embryos after hCG treatment resulted from an elevated amounts of E2.

In contrast to cyclic gilts, there were no changes in PGE2:PGFM ratio in blood plasma after hCG administration during the pregnancy. This discrepancy may be due to the different pattern of blood samples collection.

In order to explain a reason of decreased embryonic mortality in hCG treated gilts expressions of genes implicated in steroidogenesis, angiogenesis and apoptosis in the luteal tissue of these animals on day 30 of pregnancy were studied. The increased embryonic viability on day 30 of pregnancy in pigs given hCG was also demonstrated in work of Tilton et al. (1989).

The stimulatory effect of LH/hCG on steroidogenesis leads to increased expression of STAR, 3 β -hydroxysteroid dehydrogenase (HSD3 β) and P450 side chain cleavage (P450_{sc}; Rekawiecki et al. 2005). The present study demonstrated an augmented ex-

pression of STAR and LH/hCG receptor in the luteal tissue. This effect was not followed by changes in a protein concentration in the case of LH/hCG receptor. Generally, it is assumed that on day 30 of pregnancy an increase in unoccupied LH/hCG receptor (Ziecik et al. 1980) occurs due to elevated amounts of E2 originating from conceptuses (Geisert et al. 1990). Additionally, E2 seems also to up-regulate the expression of STAR, which transports cholesterol from the outer to the inner mitochondrial membrane (see: Christenson and Devoto 2003) and proclaims a crucial step in P4 synthesis. Therefore, it is supposed that this upregulation of LH/hCG receptor and STAR expression by E2 may create better conditions for P4 synthesis on day 30 of pregnancy in gilts given hCG 18 days earlier. The discrepancy between LH/hCG receptor mRNA and protein level may be associated with post-translational mechanism as was described previously (Menon et al. 2010). The lack of hCG effect on HSD3 β mRNA is less important since this enzyme is not considered as rate-limiting in P4 synthesis (Christenson and Devoto 2003). Thus, unchanged HSD3 β expression after hCG administration did not affect steroidogenesis in pregnant pigs.

The present study did not reveal any differences in mRNA expression between both isoforms of Vascular Endothelial Growth Factor (VEGF) in the luteal tissue and inter-implantation site of the endometrium after hCG administration. However, immunostaining for vWF revealed an increased angiogenesis in both the luteal and endometrial tissue of hCG-treated pigs. The expression of VEGF mRNA is up-regulated by E2 (Greb et al. 1997), while hCG favours more VEGF secretion than synthesis (Islami et al. 2003) and the observed effect could result from an early increase in VEGF expression driven by E2 and its further secretion after hCG treatment.

In the present study, the reduced percentage of CLs cells in the stage of early and late apoptosis as well as increased percentage of viable cells after hCG administration were found. So far the pathway of apoptosis activation in the porcine CL was not examined. Therefore, we cannot rule out that other molecular mechanism than Bcl-2 and Bax is triggered during the porcine CL rescuing by hCG and further detailed examinations are necessary for better understanding this process.

In summary, we confirmed that treatment with hCG on day 12 of the estrous cycle extended the lifespan of the porcine CL and prolonged P4 production. For the first time, we demonstrated that antyluteolytic action of hCG in cyclic gilts is mediated through an increased PGE2:PGF2 α ratio as well as elevated E2 in blood. Supplementation with hCG reduced embryonic mortality during the period of early pregnancy

through an enhanced angiogenesis in the luteal and endometrial tissue, and increased viability of CL cells. hCG administration also stimulates the luteal function during the pregnancy through an increased expression of LHCGR and STAR mRNA. However, further studies should be carried out to clarify the effect of hCG on whole pregnancy maintenance and litter size in pigs.

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