Praca oryginalna *Original paper*

Differential effects of progesterone, estradiol-17β, oxytocin, arachidonic acid, forskolin, and cAMP on steroid output by the porcine uterus during implantation and placentation[1)]

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Summary

The aim of the current study was to investigate: (1) secretion P_4, E_2 and A_4 by whole porcine uterine explants harvested during days 14-16 of pregnancy (implantation) and days 30-32 of pregnancy (placentation); (2) whether progesterone, estradiol, and other factors: oxytocin (OT), arachidonic acid (AA; substrate for prostaglandins synthesis) as well as forskolin (FSK; adenylate cyclase activator) and cAMP (cyclic adenosine monophosphate; second messenger) regulate the release of steroid hormones within uteri during implantation and placentation. Concentrations of P_4, E_2 and A_4 were measured in culture media by RIA. Basal uterine production of E_2 after 3-h incubation was about 2-fold higher on days 30-32 compared to days 14-16 of the pregnancy. Progesterone very strongly increased secretion of E_2 during the implantation and placentation after 3- and 24-h incubations. Control production of P_4 by uterus explants after 3-h incubation was higher during placentation than during implantation. Estradiol consistently increased the secretion of P_4 on days 14-16 and days 30-32 of the pregnancy, after 3- and 24-h incubation. The basal secretions of A_4 from uterine explants after 3-h incubation in both pregnancy periods were at the same level. Progesterone stimulated A_4 secretion on days 14-16 after 3- and 24-h. Estradiol also very strongly increased A_4 secretion during both implantation and placentation during the incubation periods. Oxytocin did not affect the release of E_2, P_4 and A_4 ($p > 0.05$) by tissue explants from either of the pregnancy periods. AA significantly stimulated E_2 secretion during implantation (after 3- and 24-h incubations) and placentation (after 3-h incubation). In contrast, AA did not effect the release of P_4 or A_4 in either of the pregnancy periods. FSK and cAMP significantly stimulated E_2 release during implantation after 3- and 24-h incubation, while during placentation only after longer incubation (24-h). The secretion of P_4 was also increased by FSK during implantation after 24-h incubation and during placentation (after 3- and 24-h incubation) as well as by cAMP in both pregnancy periods after 3- and 24-h. In addition, forskolin and cAMP very strongly stimulated A_4 release with uterine explants after 3- and 24-h incubation at the time of implantation and placentation. In summary, this is the first demonstration that: 1) the production and release of E_2 and P_4 (but not A_4) from the pig uterine explants increase with advancing gestation; and 2) P_4, E_2, arachidonic acid, forskolin and cAMP are involved in the regulation of uterine steroidogenesis, which seems to depend on the pregnancy period and incubation time.

Keywords: steroid hormones, uterus, pregnancy, pig

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Implantation is the process by which the trophectoderm of porcine blastocysts directly attaches to the endometrial luminal epithelium (1, 14). In pigs, implantation occurs around day 12 of pregnancy, following extensive tissue remodeling of the endometrium (25), where a pronounced vascularization is evident from day 13 of gestation onwards (21, 22). Porcine conceptuses secrete increased levels of estrogens, mainly 17β-estradiol (E_2), on days 11 and 12, as well as between days 14 and 18 of pregnancy (12). Estrogens enhance endometrial PGE_2 production, thus increasing PGE_2 : $PGF_{2\alpha}$ ratio necessary for corpus luteum (CL) maintenance and progesterone production (4). Implantation is completed between days 18 and 24 of pregnancy. The attachment of the embryos to the surface epithelium of the uterus initiates the placentation, as well as a rapid expansion and development of the chorion (trophectoderm) and allantois between days 18 and 30 of gestation in pigs (2). During the attachment period, interleukin (IL) (27), the transforming growth factor (TGF) beta1 (17, 18) and type I and type II interferons (19) derived from maternal and embryonic tissue, also play important roles in tissue remodeling and angiogenesis. Estrogens from the conceptuses, together with interferons, regulate endometrial gene expression. Furthermore, it has been demonstrated that porcine uterine tissues, endometrium and myometrium, synthesize and secrete steroid hormones during early pregnancy (days 14-16) and during the estrous cycle (days 14-16), and that the steroidogenesis pathway seems to be fully functional in these tissues (10, 11). However, Wojciechowicz et al. (33) showed endometrial and myometrial expression of 3β-HSD mRNA and protein as well as *in vitro* production of androstenedione (A_4) and progesterone (P_4) in pigs on days 10 to 11, 12 to 13, and 15 to 16 of pregnancy and the estrous cycle. The above *in vitro* studies concerning the pig uterus as a steroidogenic organ were performed on separated endometrial and myometrial tissue explants on days 14-16 of pregnancy. The aim of the current study was, first, to investigate the secretion of P_4, E_2, and A_4 by whole porcine uterine explants harvested on days 14-16 of pregnancy (implantation) and on days 30-32 of pregnancy (placentation) and, second, to determine whether progesterone, estradiol and other factors: oxytocin (OT), arachidonic acid (AA; substrate for prostaglandins synthesis), as well as forskolin (FSK; adenylate cyclase activator) and cAMP (cyclic adenosine monophosphate; second messenger) regulate the release of steroid hormones within uteri during implantation and placentation.

Material and methods

Experimental animals and collection of uterine tissue. All experiments were approved by the Animal Ethics Committee at the University of Warmia and Mazury in Olsztyn, Poland (AEC approval No. 66/2010/DTN). Tissue samples were recovered from mature cross-bred gilts (Large White × Polish Landrace) on days 14-16 (n = 5) of gestation (implantation) and on days 30-32 (n = 5) of gestation (placentation). The gilts were observed daily for estrous behavior and were used in the study during their third consecutive normal estrous cycle. Gilts assigned to the pregnant group were naturally bred on the second day of estrus. The animals were slaughtered at a local abattoir on days 14-16 and 30-32 of pregnancy. Pregnancy was confirmed by the presence of embryos after flushing uterine horns with 20 ml of sterile saline (days 14-16) or by the presence of morphologically-normal conceptuses (days 30-32). Uteri were immediately placed in ice-cold phosphate-buffered saline (PBS) supplemented with 100 IU/ml penicillin (Polfa, Poland) and 100 µg/ml streptomycin (Polfa, Poland) and transported to the laboratory on ice within 1 to 1.5 h for *in vitro* tissue culture.

Preparation and incubation of uterine slices. Sections of the middle part of uterine horns collected from pigs were opened longitudinally on the mesometrial surface. Uteri were washed three times in sterile PBS and then carefully cut into small pieces (400 mg weight) and washed three times in M199 medium (Sigma, USA). Individual uterine slices were placed in culture vials containing 2 ml of M199 medium supplemented with 0.1% BSA (Sigma), 20 µg nystatin (Sigma) and 20 µg gentamicin (Krka, Novo Mesto, Slovenia) and then pre-incubated *in vitro* under an atmosphere of 95% O_2 and 5% CO_2 at 37°C for 18 h. After pre-incubation, the culture medium was replaced with fresh medium and the explants were treated with vehicle (control) or P_4 (10^{-5} M; Sigma), E_2 (10^{-9} M; Sigma), OT (10^{-7} M; Sigma), AA (10^{-5} M; Sigma), FSK (10 µg/mL; Sigma) and cpt-cAMP analog (200 µM; Sigma) and incubated for an additional 3 or 24 h. All treatments were performed in triplicate.

Radioimmunoassay (RIA) of steroid hormones. Concentrations of P_4, E_2 and A_4 were measured in culture media by RIA according to the method described by Ciereszko et al. (5). The specificity of the antibodies against P_4, E_2 and A_4 has been reported by Szafranska et al. (30). Validity of the assays was confirmed by parallelism between the standard curves and a series of dilutions of randomly chosen samples. Intra-assay coefficients of variation for P_4, E_2 and A_4 assays were 4.6%, 3.7% and 0.9% respectively, while inter-assay coefficients of variation were 7.3%, 9.1% and 6.5% respectively. The sensitivities of the assays for P_4, E_2 and A_4 were 25 pg/ml, 1 pg/ml and 1 pg/ml, respectively.

Statistical analysis. All numerical data were analyzed by one-way ANOVA and a least significant difference (LSD) *post hoc* test and reported as the means ± S.E.M. from five independent observations. Statistical analyses were performed using Statistica software (StatSoft Inc., Tulsa, USA). Values for $p < 0.05$ were considered statistically significant.

Results and discussion

The present study demonstrates: 1) production and secretion of steroid hormones (E_2, P_4 and A_4) by porcine uterine tissues at the time of implantation (days 14-16) and placentation (days 30-32); and that 2) P_4, E_2, AA, FSK and cAMP are engaged in regulation of steroid secretion by uterine tissues. Basal uterine production of E_2 after 3-h incubation was about 2-fold higher (Tab. 1)

Tab. 1. Basal progesterone, estradiol, and androstenedione secretion from porcine uterine slices during implantation (days 14-16) and placentation (days 30-32)

Time of incubation	Progesterone (pg/ml)		Estradiol (pg/ml)		Androstenedione (pg/ml)	
	Days 14-16	Days 30-32	Days 14-16	Days 30-32	Days 14-16	Days 30-32
3 h	324.67 ± 28.66	501.44 ± 46.11	70.13 ± 8.14	145.23 ± 15.15	28.32 ± 3.11	26.14 ± 3.63
24 h	408.34 ± 29.34	549.13 ± 46.41	90.33 ± 8.86	172.43 ± 16.38	13.41 ± 2.09	29.31 ± 2.18

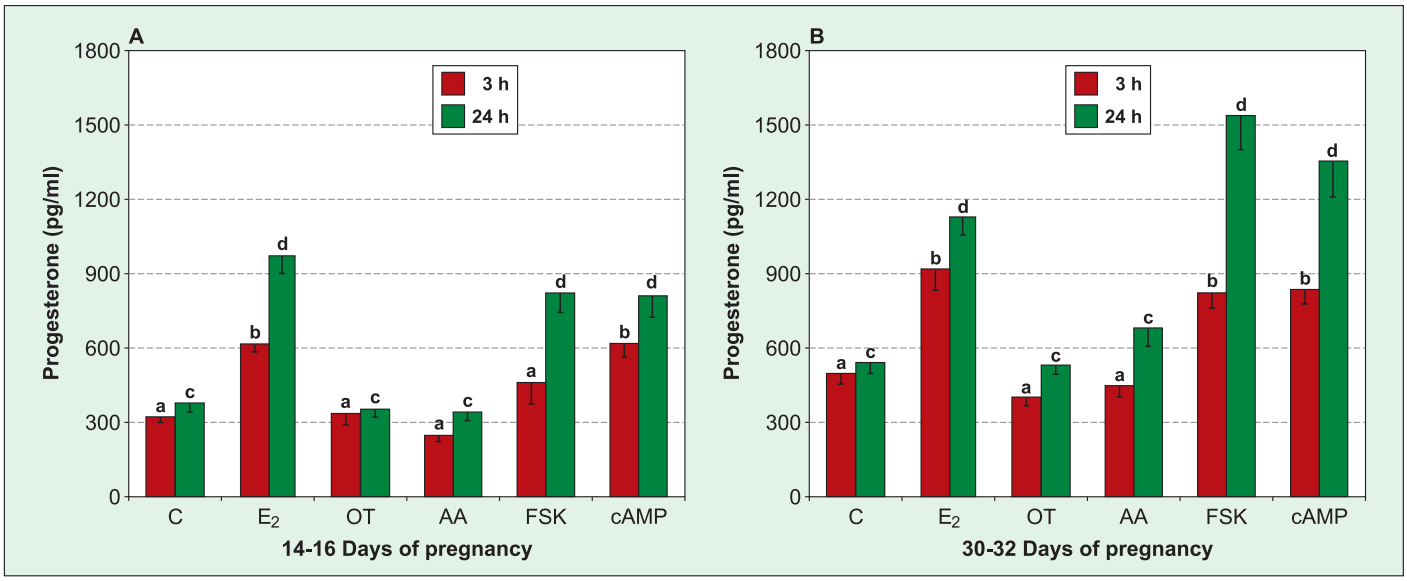

Fig. 1. The effect of P_4, OT, AA, FSK and cAMP on E_2 secretion by uterine tissue
Explanations: Release of E_2 (mean ± standard error of the mean; n = 5) *in vitro* by uterine explants of pigs on days 14-16 (A) and days 30-32 (B) of pregnancy after treatment with progesterone (P_4; 10^{-5} M), oxytocin (OT; 10^{-7} M), arachidonic acid (AA; 10^{-5} M), forskolin (FSK; 10 µg/mL), and cyclic-AMP (cAMP; 200 µM). The uterine explants (~400 mg) were preincubated (18 h; 37°C, 95% O_2 + 5% CO_2) and then incubated without (C) or with addition of experimental factors for 3 and 24 h. Different letters indicate significant differences ($p < 0.05$) between each treatment and respective control for 3- (a, b) or 24-h (c, d) incubations.

on days 30-32 (145.23 ± 15.15 pg/ml) compared to days 14-16 (70.13 ± 8.14 pg/ml) of the pregnancy. Progesterone very strongly increased secretion of E_2 (Fig. 1A and B) during the implantation ($p < 0.05$) and placentation after 3- and 24-h incubations ($p < 0.05$). The uterus tissues during the implantation period, characterized by lower basal production of the E_2, were more sensitive to exogenous P_4 than during placentation, when basal production E_2 was high. It appears that uterine sensitivity to P_4 may depend on the hormonal status of the pig as well as factors produced locally in the uterus. For instance, in the pig, trophoblast-derived estrogen production is induced by insulin-like growth factor 1 (16). There have been a limited number of reports presenting P_4 involvement in the E_2 production and secretion by the porcine uterus. A direct effect of P_4 on E_2 production by endometrial and myometrial tissues was found in our laboratory. Franczak et al. (11) showed that P_4 increased the secretion of E_2 by endometrial and myometrial slices from pregnant pigs (days 14-16) and myometrial release of E_2 was about 2-fold lower than endometrial release. It was also suggested that uterine production of estrogens may supplement the amount of steroid hormones produced by porcine embryos and may be an alternative source for this signal for pregnancy recognition in the pig (11). Estradiol and progesterone act through their receptors: the estrogen receptor (ER) and the progesterone receptor (PR), respectively. Both the PR and the ER are expressed in all compartments of the uterus and their interplay is critical to the establishment of a successful pregnancy (31). Embryo implantation depends on two important factors, embryo quality and endometrial receptivity, which are necessary for adhesion, attachment and further placentation (3). The results of the present experiment show that E_2 may also stimulate P_4 output and release by porcine uterine explants (Fig. 2A and B). Control production of P_4 by the uterine explants after 3-h incubation (Tab. 1) was higher during placentation (501.44 ± 46.11 pg/ml) than during implantation (324.67 ± 28.66 pg/ml). Estradiol consistently increased the secretion of P_4 on days 14-16 ($p < 0.05$) and days 30-32 ($p < 0.05$) of the pregnancy after 3- and 24-h incubation. The results *in vitro* obtained by Wojciechowicz et al. (33) demonstrated that the endometrial P_4 production rate is stable over the course of early pregnancy; however, endometrial production of P_4 in gravid pigs is higher than the myometrial production on days 10 to 11 and 15 to 16 of pregnancy. The authors revealed that 3β-hydroxysteroid dehydrogenase/Δ(5)-Δ(4) isomerase (3β-HSD) mRNA and protein, converting pregnenolone to P_4, are present and active in porcine endometrium and myometrium and the uterus

produces P_4 *de novo*. Previously, it was known that E_2 decreases $PGF_2\alpha$ release from the uterus into the peripheral circulation and prevents CL luteolysis (8). On the other hand, E_2 increases PGE_2 content in the porcine uterus (13) and PGE_2 secretion in endometrial tissue explants *in vitro* (32). During pregnancy, pig endometrium rapidly converts estradiol-17β to the biologically inactive estrone sulfate which is present in high concentrations within the uterine lumen of pregnant pigs (7). Our results show that the release of uterine E_2 and P_4 increases with advancing gestation and that this tendency suggests that these steroids may play an important role, for example, in proper embryo orientation and embryo-maternal communication.

The basal secretion of A_4 from uterine explants (after 3-h incubation: Tab. 1) in both pregnancy periods was at the same level: 28.32 ± 3.11 pg/ml and 26.14 ± 3.63 pg/ml, respectively. Progesterone stimulated A_4 secretion on days 14-16 after 3- and 24-h incubation ($p < 0.05$) and on days 30-32 after 3-h incubation ($p < 0.05$; Fig. 3A and B). Estradiol also very strongly increased A_4 secretion during implantation and pla-

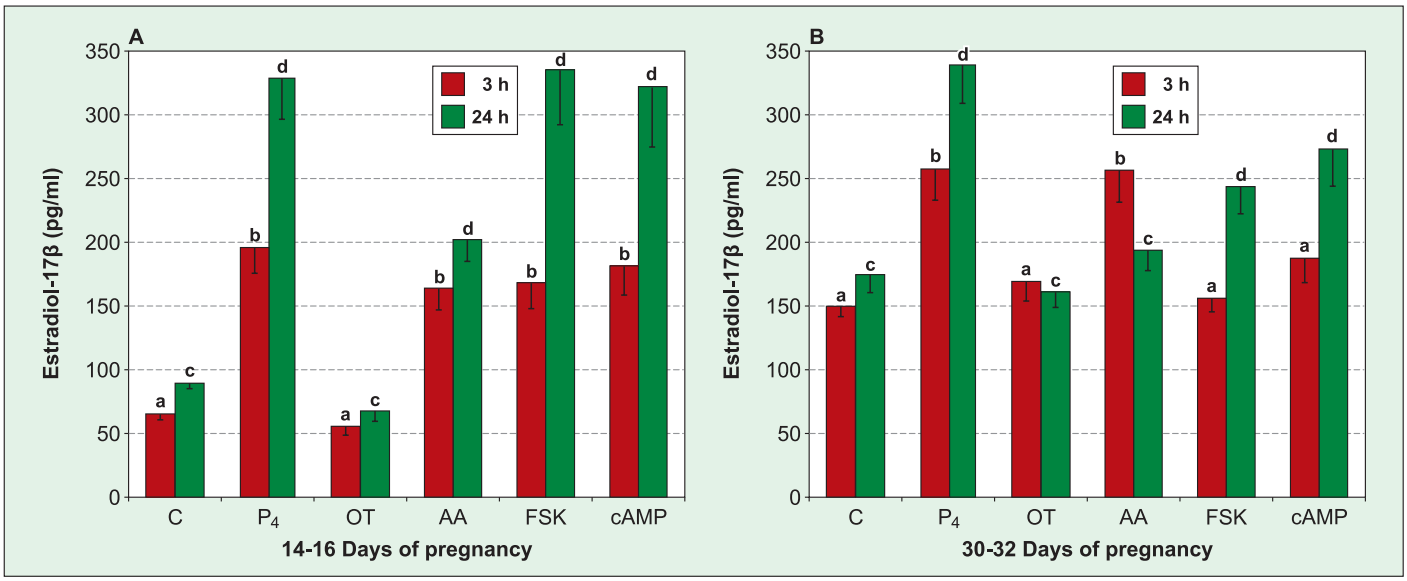

Fig. 2. The effect of E_2, OT, AA, FSK and cAMP on P_4 secretion by uterine tissue
Explanations: Release of P_4 (mean ± standard error of the mean; n = 5) *in vitro* by uterine explants of pigs on days 14-16 (A) and days 30-32 (B) of pregnancy after treatment with estradiol (E_2; 10^{-9} M), oxytocin (OT; 10^{-7} M), arachidonic acid (AA; 10^{-5} M), forskolin (FSK; 10 µg/mL), and cyclic-AMP (cAMP; 200 µM). The uterine explants (~400 mg) were preincubated (18 h; 37°C, 95% O_2 + 5% CO_2) and then incubated without (C) or with addition of experimental factors for 3 and 24 h. Different letters indicate significant differences ($p < 0.05$) between each treatment and respective control for 3- (a, b) or 24-h (c, d) incubations.

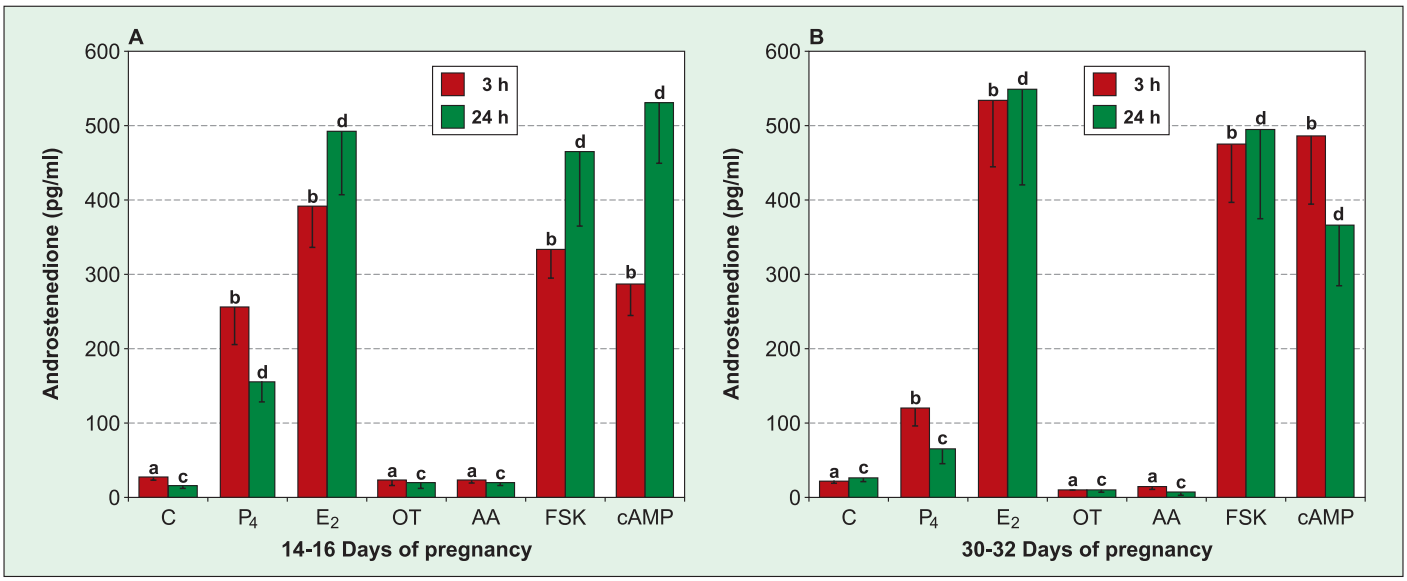

Fig. 3. The effect of P4, E_2, OT, AA, FSK and cAMP on A_4 secretion by uterine tissue
Explanations: Release of A_4 (mean ± standard error of the mean; n = 5) *in vitro* by uterine explants of pigs on days 14-16 (A) and days 30-32 (B) of pregnancy after treatment with progesterone (P_4; 10^{-5} M), estradiol (E_2; 10^{-9} M), oxytocin (OT; 10^{-7} M), arachidonic acid (AA; 10^{-5} M), forskolin (FSK; 10 µg/mL), and cyclic-AMP (cAMP; 200 µM). The uterine explants (~400 mg) were preincubated (18 h; 37°C, 95% O_2 + 5% CO_2) and then incubated without (C) or with addition of experimental factors for 3 and 24 h. Different letters indicate significant differences ($p < 0.05$) between each treatment and respective control for 3- (a, b) or 24-h (c, d) incubations.

centation during both examined incubation periods ($p < 0.05$). There is little information concerning hormonal regulation of the release of A_4 in pigs. Franczak (9) found that porcine endometrium and myometrium secrete A_4 during early pregnancy and luteolysis (days 14-16), indicating that basal endometrial and myometrial secretion of A_4 did not differ between pregnant and cyclic gilts. Progesterone increased A_4 release by both uterine tissues during early pregnancy and luteolysis. Wojciechowicz et al. (33) also indicated that endometrial and myometrial release of A_4 on days 10 to 11 did not differ from the release on days 15 to 16 in both pregnant and cyclic tissues. However, the pregnant myometrium on days 15 to 16 released more A_4 than during the estrous cycle. In pigs, A_4 is a principal circulating androgen (29) and is converted by cytochrome P450 aromatase to estrone (28). Estrone is a weak estrogen and may be reduced by 17β-hydroxysteroid dehydrogenase type 1 to E_2 (29). It is likely that uterine A_4 in pigs is a substrate for estrogen production during implantation and placentation. Furthermore, it may act in an autocrine/paracrine manner, resulting in a local increase of endometrial vascular permeability and preparation for angiogenesis, implantation and placentation.

Oxytocin did not affect the release of E_2, P_4 and A_4 ($p > 0.05$) by tissue explants from either periods of pregnancy (Fig. 1-3A and B). These results are in agreement with other studies (10, 11). However, oxytocin receptors were found in porcine endometrium during the estrous cycle and early pregnancy (24). Another report showed increased cyclooxygenase (COX-2) expression in myometrium during early pregnancy (days 14-16) and increased release PGE_2 and $PGF_{2\alpha}$ by porcine myometrium after treatment with OT (11).

The direct effects of arachidonic acid on steroid production have been reported in hen theca (20) and rat Leydig cells (26); the involvement of its metabolites in steroidogenesis has also been documented. In the present study, the results showed that AA significantly stimulated (Fig. 2A and B) E_2 secretion during implantation (after 3- and 24-h incubations: $p < 0.05$) and placentation (after 3-h incubation: $p < 0.05$). Thus the AA effect on the uterus during implantation is longer (24 hours) than at the time of placentation (only 3 hours). To infer from the present and previous evidence, it appears that estradiol can self-regulate luteotrophic PGE_2 action in the porcine uterus. In contrast, AA did not cause a release of P_4 and A_4 in either of the pregnancy periods ($p > 0.05$; Fig. 3A and B). Previously, Franczak et al. (11) showed that porcine endometrium treated with AA produced PGE_2 and $PGF_{2\alpha}$ and secreted more PGE_2 than $PGF_{2\alpha}$ during early pregnancy (days 14-16).

The literature data indicate that arachidonic acid and its lipoxygenase products are involved in the control of steroidogenesis via cAMP-mediated processes (6). FSK and cAMP significantly stimulated E_2 release during implantation after 3- and 24-h incubation ($p < 0.05$; Fig. 2 A and B) and during placentation only after longer incubation (24-h). The secretion of P_4 was also increased by FSK during implantation (after 24-h incubation: $p < 0.05$) and during placentation (after 3- and 24-h incubation: $p < 0.05$) as well as by cAMP in both pregnancy periods after 3- and 24-h ($p < 0.05$; Fig. 1A and B). In addition, forskolin and cAMP very strongly stimulated A_4 release with uterine explants after 3- and 24-h incubation ($p < 0.05$; Fig. 3A and B) at the time of implantation and placentation. An early observation indicated that forskolin increased basal and TSH-stimulated progesterone secretion by porcine luteal cells and cAMP accumulation (15) while stimulating the action of cAMP agonist on the release of progesterone and estradiol by porcine granulosa cells (23). The inverse relation was also observed: porcine endometrium tissue produced cAMP in response to PGE_2 treatment (32). However, to date little is known about the mechanism of cAMP action on uterine tissues. It is possible that the action of cAMP requires, for example, signaling pathways involving calcium and chloride ions, as well as arachidonic acid and its lipoxygenase products.

In summary, this is the first demonstration that: the production and release of E_2 and P_4 (but not A_4) from the pig uterine explants increase with advancing gestation; and that P_4, E_2, arachidonic acid, forskolin and cAMP are involved in the regulation of uterine steroidogenesis, which seems to be dependent on the period of pregnancy and incubation time.

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