

THE EFFECT OF UTERINE FLUSHINGS AND ENDOMETRIAL PROTEIN FRACTIONS ON PROGESTERONE SECRETION BY PORCINE LUTEAL CELLS

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Received April 15, 1985

Abstract

Przala J., Z. Luberd a, A. Grazul, T. Wiesak, J. Kotwica: *The Effect of Uterine Flushings and Endometrial Protein Fractions on Progesterone Secretion by Porcine Luteal Cells.* Acta Vet. Brno, 55, 1986: 163-171.

The uterine flushings (UF) were obtained from gilts on the 5th, 13th, 17th and 20th day of the cycle by washing each uterine horn with 50 ml of Eagle's medium. Additionally endometrial protein fractions (EPF) were obtained also on the 5th, 13th, 17th and 20th day of the cycle by filtration of the proteins isolated from the endometrium on Sephadex G-200.

Three EPF (I, II, III) were obtained on the 5th and 13th day of the cycle, and two (I, II) on the 17th and 20th day.

Studies were carried out on the effect of uterine flushings and endometrial fractions on progesterone (P_4) secretion by the luteal cells obtained from porcine corpora lutea on the 13th day of the estrous cycle.

Level of P_4 was determined with the RIA method after 30 min., 3 and 6-hour incubation. Levels of estradiol 17 B (E_2) and testosterone plus 5 α -dihydrotestosterone (T+DHT) in the UF were also determined, as well as level of PGF-2 α in the UF and EPF.

It was shown that concentration of PGF 2 α and E_2 was the highest on the 20th day of the cycle, and that UF from the 17th and 20th day inhibited P_4 secretion by the porcine luteal cells. Similar inhibiting effect was observed for III EPF from the 13th day of the cycle, and for I and II EPF from the 17th and 20th day of the cycle. The mentioned EPF inhibited basal production of progesterone despite the absence of immunoreactive PGF-2 α .

Pig, luteal cells, uterine flushing, endometrial fractions, progesterone.

It has been shown that luteolysis of pig corpus luteum (CL) is induced by an increase of PGF-2 α level in plasma of the uteroovarian vein (Gleeson et al. 1974; Moeliono et al. 1977). Similarly, in vitro studies revealed increased release of endometrial PGF-2 α (Patek and Watson 1976; Guthrie et al. 1978) and luteolytic effect of the endometrial extracts (Christenson and Day 1971; Watson and Mault Walker 1977). Earlier Schomberg (1967) observed also luteolytic activity of UF in pigs. Watson and Mault Walker (1977) found that UF from the late-luteal phase were more effective in inhibiting P_4 production by the luteal tissue than UF from the mid-luteal phase. Zava et al. (1980) have discovered PGF-2 α in the uterine flushings of pigs, while Murray et al. (1972) noted that the amount and quality of proteins secreted by the porcine uterus changed during the estrous cycle.

The objectives of the present study were: 1. to determine the effect of endometrial fractions obtained on the 5th, 13th, 17th and 20th day of the estrous cycle in pigs on P_4 secretion by the luteal cells, 2. to check whether isolated endometrial fractions contained immunoreactive PGF-2 α , 3. to determine the effect of UF obtained on the mentioned days of the estrous cycle on P_4 production by the luteal cells.

Materials and Methods

Animal tissues

Studies were conducted on the luteal cells obtained from the porcine corpora lutea on the 13th day of the estrous cycle. These cells are very susceptible to factors both inhibiting and intensifying P_4 secretion (Przala et al. 1984). The UF and EPF were obtained on the 5th, 13th, 17th and 20th day of the cycle from pigs for which the zero day was determined by testing the tolerance reflex. The animals were killed on the proper day, and the ovaries were collected, placed in cold sterile PBS (produced by the Laboratory of Sera and Vaccines, Lublin) with an addition of penicilin and streptomycin. Uterus was also collected and placed on ice.

Uterine flushings and endometrium

Each uterine horn was washed with 50 ml of Eagle's medium. Flushings from two horns were pooled, centrifuged and added to the isolated luteal cells ($1 \text{ ml}/5 \times 10^4$ cells). Part of UF was left in -20°C for the determination of PGF- 2α , estradiol 17β , and testosterone plus 5α -dihydrotestosterone contents. Endometrium was obtained by scraping the uterine horns with a scalpel followed by microscopic examination to see whether the myometrium has not been taken as well.

Isolation of proteins from the endometrium

The endometrium was homogenized with equal volume of 0.1 M cold NaCl and mixed for 1 hour. It was then filtered through a gauze, and absolute ethanol at -20°C was added to reach the final concentration of 86%. After 24 hours samples were centrifuged for 30 min. x 15000 g. The sediment was washed three times with acetone at -20°C . Each time the samples were centrifuged for 15 min. x 15000 g. The obtained sediment was dissolved in H_2O to final concentration of 10 mg/ml, and dialyzed for 12 hours against water. It was then centrifuged for 15 min. x 15000 g and the supernatant was salted out with ammonium sulfate for 5 hours to 40% saturation, and again centrifuged for 30 min. x 15000 g. The supernatant pH was then brought to 4.0 with 1 M acetate acid. Ammonium sulfate was added to the obtained 80% saturation solution, left for 4 hours, and then centrifuged for 30 min. x 15000 g. The supernatant was poured out, and the obtained protein sediment was dissolved in 10 ml 0.025 M acetate buffer, pH 4.0, and dialyzed against the same buffer for 12 hours. After centrifugation for 30 min. x 15000 g the obtained supernatant was transferred to a column.

Isolation of EPF

Sephadex G-200 gel filtration was used to separate the protein fractions in each sample. Sephadex columns Pharmacia Fine Chemicals (2.5×90 cm) were used to fractionate the uterine protein. The eluent was 0.025 M acetate buffer, pH 4.0, with flow rate of 20 ml/h. Gel filtration was carried out at 4°C . An aliquot of each fraction was used for protein determination, and the resulting optical density of each fraction was plotted against the elution volume to construct a protein profile. Protein fractions were lyophilized and stored at -20°C until the analysis. Concentration of the proteins was determined using the method by Lowry et al. (1951), and PGF- 2α using RIA method.

Separation of the luteal cells

Luteal cells were separated by the technique of Stouffer et al. (1976). Viable cells were counted in a haemocytometer using trypan blue stain (1%). The cells were suspended at a concentration of 5×10^4 cells/ml and placed in: 1. Leighton tubes with uterine flushings with 2% human albumin (produced by Biomed, Warsaw) and 2. Leighton tubes with Eagle's medium (Laboratory of Sera and Vaccines, Lublin) with 2% human serum albumin, and with or without 100 ng/ml of EPF. Control cultures were incubated with Eagle's medium with 2% of human serum albumin.

Incubation

The cultures were incubated at 37°C for 0.5, 3 and 6 hours. Medium samples were stored at -20°C until assayed.

Radioimmunoassays

Prostaglandin concentration in unextracted Eagle's medium samples (0.2 ml) was quantified using the system described by Cornette et al. (1972) and Kirtson (1977). The only modification of the RIA system of Kirtson (1977) was that

unbound radioactivity was measured after addition of charcoal-dextran suspension (0.625 and 0.062% respectively). Goat (# 14, pool) anti-PGF₂ α (a gift of Dr K.T. Kirton, Upjohn Co., Kalamazoo) was diluted 1:600. The antibody cross-reacted in about 54% with PGF-2α, 0.2% with 15-keto-PGF₂ α, and < 0.04% with prostaglandins of A, B and E groups. A linear regression equation ($y = -27 + 0.97x$) was calculated from the radioimmunoassayable recovery of 0.5, 1.0, 3.0 ng PGF₂ α added to Eagle's medium. Dilution of medium samples containing PGF₂ α resulted in a curve parallel to the standard curve. The smallest amount of PGF₂ α standard significantly different from zero was 12 pg at 90% confidence limit. This correspondent to a concentration of 60 pg/ml for 0.2 ml of the medium assayed. Medium blank run in the assay was negligible. Intra-assay variation determined at the concentration of 0.1-0.75 ng/ml was 7.9%.

Progesterone concentration was estimated using Abraham et al. (1971) method. Tracer [1, 2, 6, 7]-³H progesterone, (Amersham, UK) (spec. act. 87 Ci/mmol) and cold progesterone (Sigma) were used. Highly specific antibody (a - P/R₂) against progesterone was kindly supplied by prof. R. Stupnicki. Characteristics of the antibody were reported by Stupnicki (1975). Sensitivity of the assay was 128 pg progesterone/ml. Coefficients of variation within and between the assays were 5.8 and 12.5% respectively.

Estradiol 17 B was determined using the method described by Hotchkiss et al. (1971). Highly specific antibody against estradiol 17 B was kindly provided by prof. R. Rembisa. Cross-reaction for estradiol and testosterone with the antiserum was described by Przala et al. (1984). Tracer [2, 4, 6, 7]-³H estradiol - 17 B, spec. act. 96 Ci/mmol (Amersham, UK), and cold estradiol (Sigma) were used. Sensitivity of the assay was 1.9 pg/dose. Coefficients of variability within and between the assays were 6.1 and 9.7% respectively.

Testosterone plus 5α-dihydrotestosterone was determined using the method by Stupnicki (1979). Specific antibody against testosterone was kindly provided by prof. R. Rembisa. Tracer [1, 2, 6, 7]-³H testosterone, spec. act. 93 Ci/mmol (Amersham, UK) and cold testosterone (Sigma) were used. Sensitivity of the assay was 2.5 pg/dose. Coefficients of variability within and between the assays were 5.2 and 8.8% respectively.

Statistical analyses:

The results were subjected to statistical analysis, calculating the standard deviations, and significance of the differences was tested using Student's test.

Results

Differences between levels of estradiol 17 B (E₂) and testosterone plus 5α-dihydrotestosterone (T+DHT) on particular days of the cycle were not significant statistically, but the mean concentration of E₂ and T+DHT increased between the 13th and 20th day (Table 1). PGF₂ α level also increased, reaching the highest concentration in UF obtained on the 20th day of the cycle. However, immunoreactive PGF₂ α was not found in the endometrial fractions. P₄ secretion by the porcine luteal cells from the 13th day of the cycle under the effect of UF is presented in Fig. 1. It was found that UF from the 5th and 13th day of the cycle had no significant effect on P₄ secretion determined

Table 1
Concentration of PGF-2α, testosterone + dihydrotestosterone (T+DHT) and estradiol-17B (E₂) in porcine uterine flushing

Day of cycle	PGF-2α (pg/ml)	T+DHT (pg/ml)	E ₂ (pg/ml)
5	127.5 (4) ^a +56.2	140.5 (3) +49.2	168.6 (3) +37.1
13	-	255.8 (4) +83.4	212.4 (4) +45.2
17	241.6 (3) +73.1	262.5 (3) +64.2	272.2 (3) +41.2
20	574.4 (5) +94.0	222.1 (4) +61.0	373.4 (4) +68.2

Mean values ± S.E.; a - number of investigated animals

after 30 min. and 3 and 6 hour incubation. When the luteal cells were incubated with UF from the 17th and 20th day of the cycle, P_4 secretion decreased. Statistically significant differences were found in progesterone levels between the control group and the groups incubated with UF. This luteolytic effect of UF convergent with an increase of $PGF_2\alpha$ and E_2 .

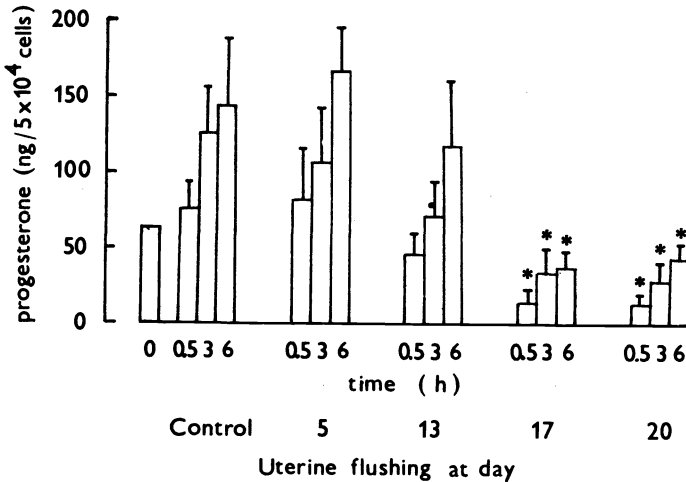


Fig. 1

The effect of uterine flushings from the 5th, 13th, 17th and 20th day of the estrous cycle on progesterone secretion by the porcine luteal cells from 13th day of the cycle. Mean values \pm S.E. for 5 gilts/group. *Difference significant at $p < 0.05$ (t-test).

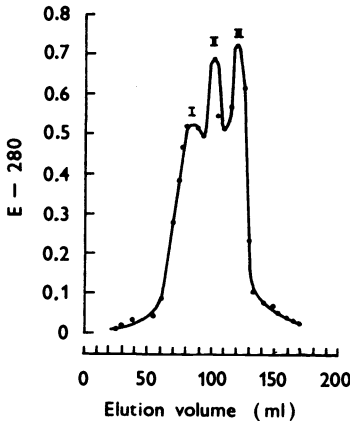


Fig. 2

Fractionation of precipitate of the porcine endometrial extract from 5th day of the cycle on Sephadex G-200. Fractions I-III.

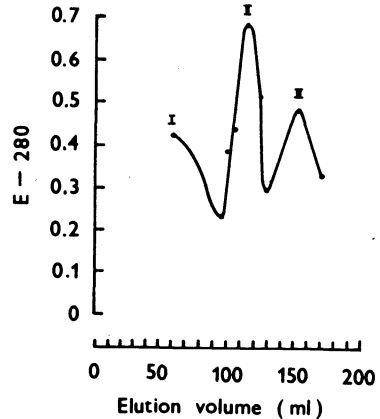


Fig. 3

Fractionation of precipitate of the porcine endometrial extract from 13th day of the cycle on Sephadex G-200. Fractions I-III.

EPF on the 5th, 13th, 17th and 20th day of the cycle, and protein concentrations are presented in Figs. 2, 3, 4, 5 and Table 2. On the 5th and 13th day of the cycle three fractions were obtained (I-III), while on the 17th and 20th day two fractions (I-II). Also protein concentration in the endometrial fractions was fairly differentiated. The highest protein content was noted in fraction III on the 5th day of the cycle, the lowest in fraction II on the 17th and 20th day (Table 2).

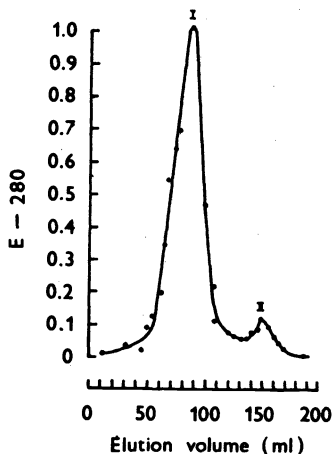


Fig. 4.

Fractionation of precipitate of the porcine endometrial extract from 17th day of the cycle on Sephadex G-200. Fractions I-II.

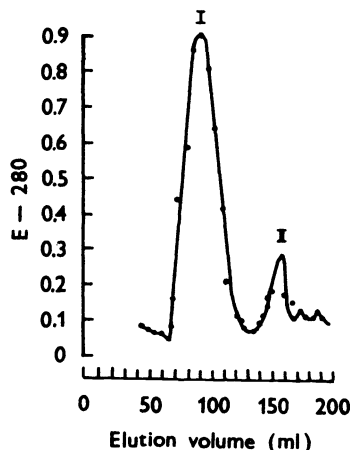


Fig. 5.

Fractionation of precipitate of the porcine endometrial extract from 20th day of the cycle on Sephadex G-200. Fractions I-II.

Table 2.
Protein fractions from the porcine endometrium at different days of the cycle

Day of the cycle	Fractions	Protein concentration mg/100 mg of extract
5	I	21.70
	II	25.20
	III	30.45
13	I	17.50
	II	27.30
	III	19.00
17	I	24.80
	II	4.20
20	I	26.20
	II	4.20

Studies on the effect of endometrial fractions upon the steroidogenesis revealed that the endometrial fractions from the 5th day of the cycle did not affect progesterone secretion by the luteal cells during 6 hour incubation. The only trend noted was a certain lowering of the hormone level during incubation of the luteal cells in the presence of fraction III (Fig. 6). Similar results were obtained during incubation of the luteal cells with fraction I and II from the 13th day of the cycle. However, progesterone levels in the samples incubated with fraction III were significantly lower ($P < 0.05$) than in the control group (Fig. 7). Endometrial fractions from the 17th and 20th day of the cycle had a most pronounced effect on progesterone secretion (Figs. 8 and 9). It was shown that fractions I and II inhibited ($P < 0.05$) secretion of this steroid during 6 hour incubation.

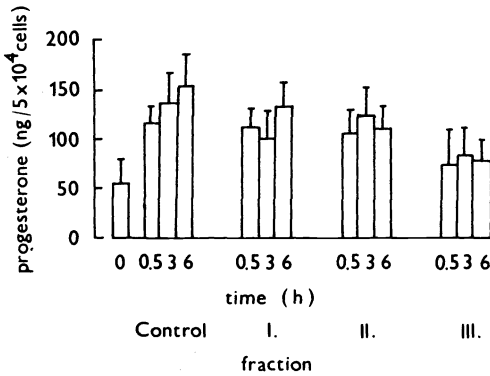


Fig. 6. The effect of protein fraction (100 ng/ml) from the 5th day of the estrous cycle on progesterone secretion by the luteal cells on 13th day of the cycle. Mean values \pm S.E. for 4 gilts/group.

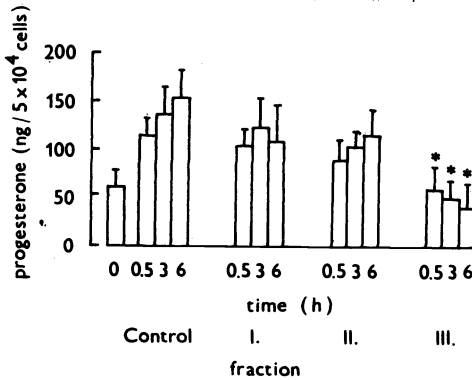


Fig. 7. The effect of protein fraction (100 ng/ml) from the 13th day of the estrous cycle on progesterone secretion by the luteal cells on 13th day of the cycle. Mean value \pm S.E. for 4 gilts/group. *Difference significant at $P < 0.05$ (t-test).

Discussion

Concentration of estradiol and $\text{PGF}_2\alpha$ in the porcine uterine flushings was previously studied by Z a v a y et al. (1980). These authors found that content of estradiol 17β in the uterine flushings increased between 10th and 18th day of the estrous cycle, while $\text{PGF}_2\alpha$ content reached a maximum on the 18th day of the cycle. The present studies took into account also the 20th day of the cycle and revealed that estradiol and $\text{PGF}_2\alpha$ content in the uterine flushings was higher on this day than on the 17th day of the cycle (Table 1). The uterine flushings contained twice as much $\text{PGF}_2\alpha$ on the 17th day than on the 5th day, and 4 times more on the 20th day. Hence, it can be suggested that $\text{PGF}_2\alpha$ present in the uterine flushings from the late-luteal phase inhibited progesterone production. This inhibition was observed notwithstanding increased estradiol concentration, which normally prevents luteolysis of the corpora lutea in pigs (K i d d e r et al. 1955; G a r d n e r et al. 1963; F r a n k et al. 1978).

Endometrial fractions affected progesterone secretion by the luteal cells in the same way as the uterine flushings. Fraction III from the 13th day of the cycle, and fractions I and II from the 17th and 20th day had a luteolytic effect. This effect was noticed despite the absence of immunoreactive $\text{PGF}_2\alpha$ in the endometrial fractions. Most probably, chemical procedure of isolating the endometrial fractions inactivated $\text{PGF}_2\alpha$, as this substance is very labile. Measurements of steroid levels in the endometrial fractions from the 13th day of cycle revealed lack of estradiol 17β , while progesterone and testosterone were both present. Progesterone concentration amounted to 2.0 ng/100 ng of protein in fraction I, and to 2.5 and 1.6 ng in fractions II and III respectively.

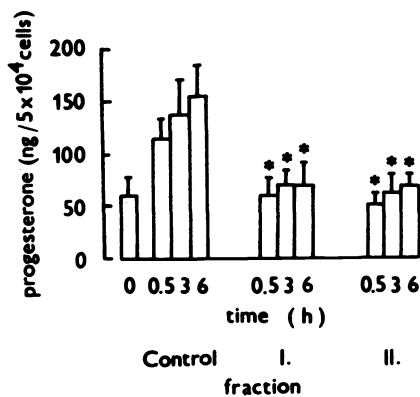


Fig. 8.

The effect of protein fraction (100 ng/ml) from the 17th day of the estrous cycle on progesterone secretion by the luteal cells at 13th day of the cycle. Mean values \pm S.E. for 4 gilts/group. *Difference significant at $P < 0.05$ (t-test).

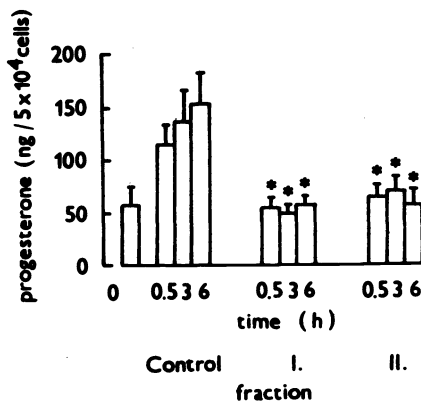


Fig. 9.

The effect of protein fraction (100 ng/ml) from the 20th day of the estrous cycle on progesterone secretion by the luteal cells at 13th day of the cycle. Mean values \pm S.E. for 4 gilts/group. *Difference significant at $P < 0.05$ (t-test).

Testosterone levels amounted to 24, 68 and 30 pg/100 ng of protein respectively in fractions I, II and III. It can be assumed that exogenous progesterone and testosterone present in the endometrial fractions at rather low levels had no significant effect on endogenous secretion of this hormone. Studies carried out on granulosa cells in rats revealed that testosterone and 5 α dihydrotestosterone acted synergistically with FSH, stimulating cholesterol transformation into progesterone (Nimrod and Lindner 1976; Armstrong and Dorrington 1976; Leung et al. 1979). At the same time, the present studies revealed that none of the endometrial fractions containing testosterone stimulated progesterone secretion.

We have not been able to find any works dealing with the effect of endometrial protein fractions on progesterone secretion. Murray et al. (1972) isolated protein fractions from the uterine flushings. These authors also obtained three protein fractions until the 9th day, but in later phases of the cycle additional fractions IV and V appeared. These fractions were not present before the 9th and after the 16th day of the cycle.

Differences in the number of protein fractions obtained by Murray et al. (1972) and found in our studies might have resulted from different biological material used, or from different procedure of separating the uterine flushings and the proteins isolated from the endometrium. Concentration of proteins in the uterine flushings studied by Murray et al. (1972) reached the peak on the 15th day of the cycle, and then decreased rapidly, so that on the 17th, 18th and 20th day it was similar as before the 10th day. Our studies also revealed different level of proteins in the endometrial fractions. The highest concentration of proteins was observed in fraction III on the 5th day, and in fraction II on the 13th day of the cycle. The lowest protein concentration was noted in fraction II on the 17th and 20th day of the cycle (Table 2). Hence, a question arises whether addition of the endometrial fractions with different protein levels into the incubated luteal cells affected progesterone secretion. Taking into account that fraction I on the 17th and 20th day of the cycle contained about 6 times more protein than fraction II, whereas its effect on progesterone secretion was similar, it could be assumed that protein levels did not affect the results of the experiments. On the other hand, it can be suggested that the process might have been affected by the type of protein produced by the endometrial tissue, or by the presence of some other factor(s) with luteolytic effect. Studies by Squire et al. (1972) revealed that the electrophoretic pattern of proteins changed during the estrous cycle in pigs. The protein components of porcine uterine secretions showed similar electrophoretic pattern on day 2-11. Beginning on day 12 there were additional protein bands in the postalbumin area.

Влияние утеринных выделений и эндометриальных белковых фракций на секрецию прогестерона лютеальными клетками свиной

Od prasníček 5., 13., 17. a 20. den pohlavního cyklu byly získávány výplašky uteru výplachem každého děložního rohu 50 ml Eaglova média. Filtrací proteinů izolovaných z endometria na Sephadexu G-200 byly získány endometriální proteinové frakce (EPF) prasníček ve stejných dnech cyklu.

Tři EPF (I., II. a III) byly získány 5. a 13. den cyklu, dvě frakce (I., II) 17. a 20. den cyklu.

Byl sledován vliv uterinních výplašků a endometriálních proteinových frakcí na sekreci progesteronu (P_4) luteálními buňkami získanými z corpora lutea prasníček 13. den cyklu.

Koncentrace P_4 byla stanovena pomocí RIA metody po inkubaci 30 minut, 3 a 6 hodin. Hladiny estradiolu 17β (E_2), testosteronu plus 5 α dihydrotestosteronu (T+DHT), a PGF-2 α byly stanoveny jak ve výplacích, tak i v endometriálních proteinových frakcích.

Bylo prokázáno, že koncentrace PGF-2 α a E_2 byla nejvyšší 20. den cyklu, a že uterinní výplašky získané 17. a 20. den inhibovaly sekreci P_4 luteálními buňkami prasníček. Podobný inhibiční účinek měla i EPF od 13. dne cyklu, a frakce I. a II. 17. a 20. den cyklu. Endometriální proteinové frakce inhibovaly základní produkci progesteronu i v nepřítomnosti imunoreaktivního PGF-2 α .

Влияние маточных экссудатов и эндометриальных белковых фракций на секрецию прогестерона лютеальными клетками свиной

Маточные экссудаты (МЭ) извлекали путём промывания каждого рога матки свинок 50 мл раствора Игла на 5, 13, 17 и 20 день эстрального цикла. Эндометриальные белковые фракции (ЭБФ) также получали на 5, 13, 17 и 20 день цикла путём гель-фльтрации на сефадексе G-200 белков, изолированных из эндометрия.

На 5 и 13 день цикла получили три (I, II, III) ЭБФ, а на 17 и 20 день - две (I, II) ЭБФ.

Исследовали влияние маточных экссудатов и эндометриальных фракций на секрецию прогестерона (P_4) лютеальными клетками, полученными из жёлтых тел свинок на 13 день цикла.

Уровень P_4 определяли радиоиммунологическим методом по истечении 30 минут, а также 3 и 6 часов инкубации. Кроме того, определяли уровень эстрадиола 17β (E_2) и тестостерона плюс 5 α -дигидротестостерона (T+DHT) в МЭ, а также уровень простагландина F-2 α (PGF $_2$, α) в МЭ и ЭБФ.

Констатировали, что концентрация PGF $_2$, α и E_2 была наиболее высокой на 20 день цикла и что МЭ с 17 и 20 дня тормозят секрецию P_4 лютеальными клетками свиной. Подобное тормозящее действие оказывала III ЭБФ с 13 дня цикла, а также I и II ЭБФ с 17 и 20 дня цикла. Перечисленные ЭБФ тормозили базальную продукцию прогестерона, несмотря на отсутствие иммунореактивного PGF-2 α .

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Acknowledgements

Appreciation is expressed to dr. J.W. Landerdale and K.T. Kirton, Upjohn Co., Kalamazoo, for PGF₂ α and goat anti-PGF₂ α, to prof. R. Rembiesa, Institute of Pharmacology, Polish Academy of Sciences, Kraków, for donating testosterone and estradiol 17β antiserum, and to prof. R. Stupnicki, Polish Academy of Sciences, Jablonna, for providing progesterone antiserum.

This study was supported financially by the Institute of Pathology and Animal Therapy, Agricultural Academy, Wrocław (MR-II-10).