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CORTISOL, PROGESTERONE AND ESTRADIOL SECRETION *IN VITRO* IN POSTPARTUM PLACENTAL COTYLEDONS OF EWES THAT GAVE BIRTH TO SINGLE OR TWIN LAMBS

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Abstract

The level of fetal hypothalamic-pituitary-adrenal (HPA) axis activity is determined by gestation length, which can be influenced by an adverse intrauterine and/or maternal environment and also by litter size. This study was designed to determine whether steroid hormone secretion by ewe placenta is related to single or twin pregnancy. Placentas expelled from twelve postpartum Polish Longwool sheep were used. Six of them gave birth to a single lamb (SPC) and the other six delivered twin lambs (TPC). Cotyledons were removed from the placenta and their slices were incubated in TCM-199 medium supplemented with 10% FSC and antibiotics under an atmosphere of 5% CO_2 at 37°C. After incubation of placental explants, the level of progesterone (P_4), estradiol (E_2), and cortisol was measured in medium by RIA. Gestation length in the SPC and TPC ewes was 145.2±0.75 and 142.8±0.4 days, respectively. After 1 and 3 h of incubation, higher concentration of P_4, E_2 ($P \leq 0.01$) and cortisol ($P \leq 0.05$) was observed in the TPC compared to the SPC group, in which mean gestation length was 2.34 days longer. The observed changes indicate that in the ewe, the in vitro secretion of placental steroid hormones is related to the length of pregnancy.

Key words: ewe placenta, pregnancy, cortisol, progesterone, estradiol

Placental hormones are required for the establishment and maintenance of pregnancy, adaptation of the maternal organism to pregnancy, growth and well being of the fetus, as well as development of the endocrine mechanisms involved in parturition (Challis et al., 2001; Whittle et al., 2001).

It has been more than 40 years since the involvement of the hypothalamic-pituitary-adrenal (HPA) axis in the initiation of parturition in sheep was discovered (Lig-

gins et al., 1967). Numerous studies have demonstrated the importance of this endocrine axis as a central feature of the fetal stress response, as well as for coordinating readiness for birth with the timing of birth (Wood, 2005; Challis et al., 2001; Whittle et al., 2001). In the late phase of gestation the fetus triggers the onset of parturition through increased activity of HPA axis. There are increases in the concentration of ACTH followed by a rise of cortisol secretion in the fetal circulation. The increase of adrenal cortex sensitivity to ACTH also induces placental estrogen production. The interaction between estradiol and the fetal HPA axis might function as a positive feedback loop that enables an increase in concentrations of estradiol and cortisol before birth (Pasqualini, 2005; Wood, 2005; Whittle et al., 2001).

During the last 15–20 days of gestation fetal cortisol concentration rises towards term and also increases activation of the glucocorticoid-dependent enzymes involved in placental estrogen synthesis (Pasqualini, 2005; Wood, 2005; Whittle et al., 2001; Burton and Waddell, 1999). Progesterone is required throughout gestation to maintain pregnancy in ewes. Concentration of circulating progesterone in jugular venous blood in sheep increases from day 55, with half of it being from corpus luteum and half being from the placenta at day 90 of pregnancy, peaking around day 130, and declining before parturition (Weems et al., 2006; Weems et al., 1992). The prepartum rise in fetal cortisol had an effect on increased P450 C_{17} activity in ovine placenta. As a result, C_{21} steroids (pregnenolone, progesterone) reaching the placenta could be metabolized to C_{19} steroids decreasing placental progesterone production. When the progesterone:estradiol-17β ratio is decreased, pregnancy is threatened (Challis et al., 2001; Weems et al., 2007).

The growth of placenta and individual placentomas is influenced by maternal nutrient status and by litter size (Mellor, 1983). Number of fetuses influences gestation length which is extremely stable for a particular sheep breed. However, single-bearing sheep have a longer gestation period by 1 to 2 days than twin-bearers (Forbes, 1967; Gordon, 1967). Moreover, the shorter course of gestation in twin pregnant ewes may occur because of precocious activation of HPA axis when fetuses are exposed to an adverse intrauterine environment, such as hypoxemia (Challis et al., 2001).

The objective of this study was to investigate whether the concentration of cortisol, estradiol and progesterone of the ewe placenta incubated *in vitro* after birth is related to singleton or twin pregnancies.

Material and methods

Twelve ewes of Polish Longwool sheep, 2 to 3 years of age with a mean body weight of 60±5 kg were used in this study. They were randomly chosen from the flock after delivery and divided into 2 groups. Group 1 consisted of six females (n = 6) which gave birth to single lambs (single placental cotyledons, SPC) and group 2 contained 6 ewes (n = 6) which delivered twins (twin placental cotyledons, TPC). The ewes were kept in a sheepfold of the Research Station belonging to the

Sheep and Goat Breeding Department of the University of Agriculture in Kraków (Bielany). The ewes were fed twice daily with hay and grass silage and water *ad libitum*. Gestation length was recorded in the SPC and TPC groups. All animal care and experimental procedures were reviewed and approved by the Local Ethics Committee for Animal Experimentation.

Immediately after birth, placentas of groups SPC and TPC were collected and cotyledons explanted, weighed and rinsed 5 times in phosphate buffered saline (PBS) (Biomed, Lublin) supplemented with antibiotics (240 IU penicillin and 0.2 mg streptomycin/ml; Pliva, Kraków). Afterwards, cotyledons were rinsed again 3 times in TCM-199 medium (Biomed, Lublin) supplemented with antibiotics (120 IU penicillin and 0.1 mg streptomycin), 10% FCS (Fetal Calf Serum; Biomed, Lublin) and Red Blood Lysing Buffer (1:70, Sigma, USA). Tissue slices of cotyledons were weighed, diced and distributed at 20 mg in 1 ml of culture medium into each of 24 wells of the plastic tissue culture dish. Culture medium was composed of TCM-199 medium with 10% FSC and Antibiotic Antimycotic Solution (1:200; Sigma, USA). Incubation during 1, 3 and 6 hours was performed in a 5% CO_2 humidified atmosphere in air, at 37ºC. Medium was collected, changed after 1, 3 and 6 h of incubation and stored frozen (–20°C) until analysed.

All investigated steroids were measured using appropriate RIAs Spectra kits (Orion Diagnostica, Finland).

Cortisol

The limit of assay sensitivity was 5 nmol/l. The coefficients of variation within and between assays were 3.56% and 6.76%, respectively. The cross-reactivity of the cortisol antiserum with 5-α-dihydrocortisol was 84.3%. Other steroids tested showed cross-reactivity from 78.8% (21-deoxy-cortisol) to less than 1% (aldosterone, cortisone).

Progesterone

The limit of assay sensitivity was 0.3 nmol/l. The coefficients of variation within and between assays were 4.3 and 4.96%, respectively. The cross-reactivity of the progesterone antiserum with pregnenolone was 3.9%. All other tested steroids (corticosterone, 5β-dihydroprogesterone, testosterone and estriol) showed less than 1% cross-reactivity.

Estradiol

The limit of sensitivity was 30 pmol/l. The coefficients of variation within and between assays were 5.25% and 3.7%, respectively. The cross-reactivity of the estradiol antiserum with ethinyloestradiol was 1.4%. All other steroids tested (estron, estriol, progesterone) showed less than 1% cross-reactivity.

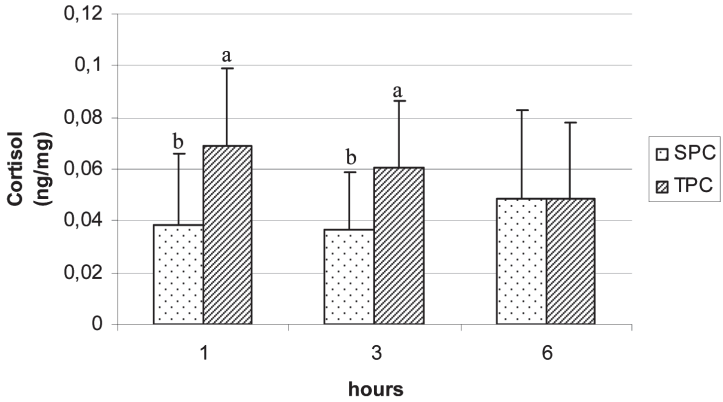
Statistical analyses

Analysis of variance (ANOVA, SAS statistical package) and Student t-test were used to compare the mean hormone concentration in incubates of placental explants from single and twin pregnancies.

Results

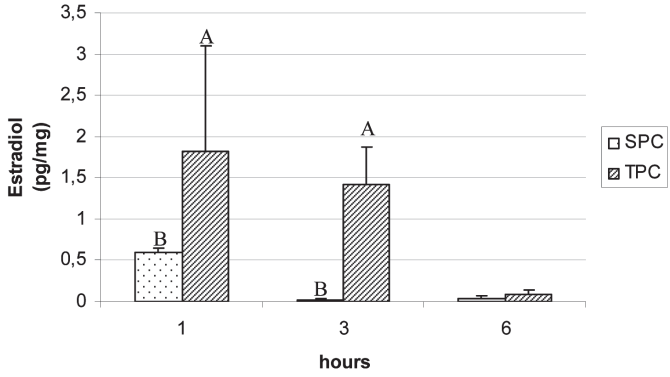
Gestation length of the ewes was 145.2±0.75 and 142.8±0.4 days in the SPC and TPC groups, respectively. The difference in mean gestation length between the groups was 2.34 days.

Cortisol concentration in culture medium was higher in the TPC group than in the SPC group (P≤0.05) after 1 and 3 h of incubation but did not differ at 6 hours (Figure 1).



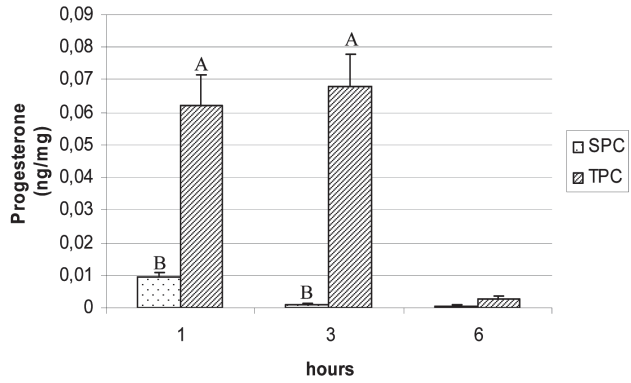
a, b – different superscripts show statistically significant differences (P≤0.05).

Figure 1. Mean (±SD) cortisol concentration in slices of postpartum placental cotyledons incubated *in vitro* (ng/mg wet weight) from ewes that gave birth to single (SPC) and twin (TPC) lambs



A, B – different superscripts show statistically significant differences (P≤0.01).

Figure 2. Mean (±SD) estradiol concentration in slices of postpartum placental cotyledons incubated *in vitro* (ng/mg wet weight) from ewes that gave birth to single (SPC) and twin (TPC) lambs



A, B – different superscripts show statistically significant differences (P≤0.01).

Figure 3. Mean (±SD) progesterone concentration in slices of postpartum placental cotyledons incubated *in vitro* (ng/mg wet weight) from ewes that gave birth to single (SPC) and twin (TPC) lambs

A significantly (P≤0.01) higher level of E_2 in culture medium after 1 and 3 h of incubation was found in the SPC group compared to the TPC group. A meaningful decrease of E_2 content in medium was observed after 6 h of incubation in both groups (Figure 2).

The difference in P4 content in the analysed media was striking between the SPC and TPC groups (P≤0.01) (Figure 3). P_4 level in the SPC group was low after 1 h and decreased to a negligible level during the next 3 h of incubation. On the other hand, a high level of P_4 was measured in conditioned medium after 1 and 3 h of TPC explant incubation. After 6 h progesterone amount was measured within limits of the assay used in both groups.

Discussion

The present study has demonstrated that TPC secreted higher amounts of cortisol *in vitro* than SPC. It has been previously shown that the prepartum surge of cortisol occurs later in twin compared with singleton sheep fetuses in late gestation. This is a consequence of the fetal HPA axis maturation which is delayed in twin compared with singleton sheep fetuses during late gestation (Edwards and McMillen, 2002; Gardner et al., 2004). Thus we speculate that because of more and more uncomfortable intrauterine condition twin fetuses compete for placental substrate supply in late gestation (Murawski et al., 2010), and by then a programmed delay in the prepartum increase of cortisol concentration in blood plasma in twin fetal sheep would protect against preterm delivery (McMillen et al., 2004). However, our results show that a higher level of cortisol in TCP could be the reason for shorter gestation length of twin pregnancy compared to SCP.

In spite of the fact that cortisol is not synthesized by sheep placenta (Burton and Waddell, 1999), we found low concentration of cortisol in culture medium at 1, 3 and 6 h of incubation in SPC and TPC (Figure 1). The presence of cortisol in placenta can be explained by its penetration into placenta from mother or fetus blood circulation and then release to culture medium during incubation.

During mammalian pregnancy, except rodents, the concentration of cortisol in mother's blood is much higher than that in the fetus (Whittle et al., 2001). In sheep 90% of cortisol in the fetal circulation is of maternal origin at the time when fetal adrenals begin cortisol production, i.e. close to term, and then fetus becomes the primary source of circulating glucocorticoids (Burton and Waddell, 1999). Between the mother and her fetus, only placenta is a barrier apart from uterus which prevents excess of maternal cortisol crossing into the fetus (Whittle et al., 2001). At the end of pregnancy cortisol predominates, which is explained by the important biological role of this hormone during the prenatal period (Alfaidy et al., 2003; Liggins et al., 1967). The importance of cortisol/cortisone ratio in fetal development, during the course of pregnancy, parturition and postnatal life is well known.

In ruminants, placental estrogen biosynthesis is strongly influenced if not controlled by the fetal HPA axis (Whittle et al., 2001). The secretion of cortisol by fetal adrenal glands increases fetal plasma cortisol, which in turn induces the activity of CYP17 (cytochrome P450c17). The induction of CYP17 is a critical step in the pathway to increasing placental production of estrogen from progesterone and pregnenolone. Increasing estrogen biosynthesis by the placenta as a result of increasing fetal HPA axis activity, stimulates fetal pituitary to increase ACTH secretion. The positive feedback loop of estradiol with the fetal HPA axis augments the increase of its activity in the final days of fetal life and results in the initiation of labour (Wood, 2005). So far the information about rate of estradiol secretion by explants of placental cotyledons *in vitro* is scarce. However, our observations suggest that higher ability for estradiol secretion after 1 and 3 h of explant incubation is shown by TPC compared to SPC.

The results presented by Tuckey et al. (1994) showed that freshly isolated human trophoblasts maintain a rate of progesterone synthesis that is not appreciably stimulated by cholesterol sulphate (a more soluble form of cholesterol which can readily enter placental cells and supply P450scc with substrate) until 3 h of incubation. Thus, immediately after isolation from the placenta, these cells have near-saturating cholesterol supply for progesterone synthesis which soon becomes a limiting factor. Similar data obtained in this study confirm that the high amount of progesterone was secreted only after 1 and 3 h of incubation in both SPC and TPC groups, decreasing to a very low level at 6 h.

It is difficult to explain why concentrations of measured estradiol and progesterone were higher in TPC than in SPC. It can be supposed that cells of TPC have a higher ability for hormone secretion in comparison with SPC. This may have significant influence on postnatal adaptation of progeny to environmental conditions. On the basis of results published by Budge et al. (2003), Edvards and McMillen (2002), Challis et al. (2001) and Medrano and Bradford (1991), it may be speculated that a higher level of placental hormone secretion could result from poorer fetal develop-

ment. However, significantly higher amounts of estradiol and progesterone produced by TPC could be the result of shorter gestation length (by 1 to 2 days), which could make a larger number of placental cells sustain their biological lifespan compared to the cells of SPC animals which carried one fetus and had pregnancy 1 to 2 days longer (Forbes, 1967; Gordon, 1967). In our study, gestation in the SPC group was 2.34 days longer than in the TPC group. This may suggest that cells of placenta explants of TPC after adaptation to the incubation conditions were still able to synthesize estradiol and progesterone in a way they did it *in vivo*. Since placenta is a transient organ, it seems possible that in single pregnancy a larger number of placenta cells terminate their biological lifespan than in twin pregnancy which is shorter. These results were obtained for the first time, and were used as a basis for elucidation of the cause of existing differences in placental estradiol and progesterone hormone secretion between SPC and TPC *in vitro*.

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Koncentracja kortyzolu, progesteronu i estradiolu *in vitro* w postnatalnych liściach łożysk owiec, które urodziły jedynaki lub bliźnięta

STRESZCZENIE

Poziom aktywności osi podwzgórzowo-przysadkowo-nadnerczowej (HPA) jest zależny od długości ciąży, która może być modyfikowana przez niekorzystne wewnątrzmaciczne i/lub matczyne środowisko, a także przez wielkość miotu. Badania zaplanowano w celu określenia, czy sekrecja hormonów steroidowych przez łożysko owcy jest uzależniona od jedynaczej lub bliźniaczej ciąży. W badaniach wykorzystano wydalone tuż po porodzie łożyska od dwunastu owiec rasy polskiej długowełnistej. Sześć z tych łożysk pochodziło od owiec, które urodziły jedynaki (SPC), a sześć kolejnych od owiec, które urodziły bliźnięta (TPC). Z łożysk izolowano liścienie, a ich skrawki inkubowano w pożywce TCM-199 z dodatkiem 10% FSC i antybiotyków w atmosferze z dodatkiem 5% CO_2 i temp. 37°C. Po inkubacji eksplantów łożyska, w pożywce mierzono poziom progesteronu (P4), estradiol (E2) i kortyzolu metodą RIA. Długość ciąży u owiec w grupie SPC i TPC wynosiła odpowiednio 145,2±0,75 i 142,8±0,4 dnia. Po 1. i 3. godzinie inkubacji wyższą koncentrację P_4, E_2 ($P\leq0,01$) i kortyzolu ($P\leq0,05$) obserwowano w grupie TPC niż SPC, w której średnia długość ciąży była o 2,34 dnia dłuższa. Powyższe obserwacje wskazują na zależność pomiędzy długością ciąży a poziomem sekrecji steroidowych hormonów łożyskowych u owcy.