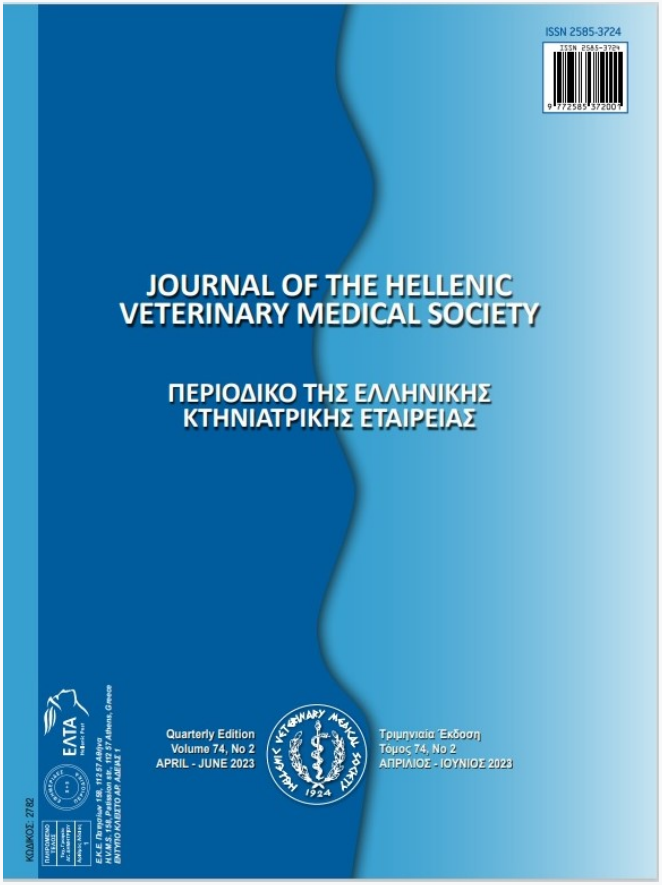
Journal of the Hellenic Veterinary Medical Society

Vol 74, No 2 (2023)



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doi: 10.12681/jhvms.26611

To cite this article:

Özalp, G., Ortaç , C., Bozkurt, B., Rişvanlı, A., Aktar , A., Yavuz, A., Korlu, Y., & Şeker, İ. (2023). Expression of steroidogenic enzymes in placentome of ewes with pregnancy toxemia after two parturition induction methods. *Journal of the Hellenic Veterinary Medical Society*, *74*(2), 5505–5516. https://doi.org/10.12681/jhvms.26611

J HELLENIC VET MED SOC 2023, 74 (2): 5505-5516
ΠΕΚΕ 2023, 74 (2): 5505-5516

Research article
Ερευνητικό άρθρο

Expression of steroidogenic enzymes in placentome of ewes with pregnancy toxemia after two parturition induction methods

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ABSTRACT: The regulation pattern of important enzymes in placental steroidogenesis and prostaglandin production in ewes with pregnancy toxemia is reviewed. The alterations of gene expressions after the administration of aglepristone (AG) and dexamethasone (DEX) are also discussed. Four healthy (CG) and 22 ewes with experimental pregnancy toxemia were included in the study. Ewes with pregnancy toxemia of group AG (n=9) and group DEX (n=9) were injected twice with 10 mg/kg of aglepristone and once with 5 ml dexamethasone respectively to induce parturition on 141±1,3 day of gestation; whereas healthy control [Group CG (n=4)] and pregnancy toxemia [Group PT (n=4)] group received no treatment for parturition induction. Placentomes were immediately collected right after the expulsion of the last lamb. mRNA extraction from total placentome capsule, cotyledon and caruncle was carried out and Real-Time PCR was performed. Serum samples were collected from ewes and cortisol, PGFM, PGE_2, estrone sulfate and progesterone concentrations were measured after treatments until parturition. The lowest mRNA expressions of steroidogenic enzymes were detected in group PT. Interestingly expression pattern of steroidogenic enzymes in group AG was similar to group PT. No difference was found in mRNA expressions of 3βHSD and CYP19 among groups. Between groups, AG-DEX the mRNA expressions in the caruncle of $PTGS_2$/COX_2 and PGFS were statistically different respectively ($P<0.005$). A significant difference could be observed in EP3 expression in the caruncle of DEX and AG compared to CG ($P<0.05$); however PTGES, EP1, EP2, and EP4 expressions were not statistically different among groups ($P>0,05$).

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Date of initial submission: 30-03-2021
Date of acceptance: 25-02-2023

Estrone sulfate, $PGE_{,2}$ and PGFM concentrations were statistically different, however, no difference was observed in cortisol levels between groups. The present study suggests that the endocrinologic pathway controlling parturition is different in ewes with pregnancy toxemia. Dexamethasone administration endocrinologically mimicked normal parturition, but the genes regulating uterine contractions were similarly expressed, as in group PT. Probably expressions of EP1 and tissue-specific counter-expressions of cervical EP genes could refer to the pathogenesis of insufficient cervical dilatation, observed in pregnancy toxemia and dexamethasone applications.

Keywords**:** Prostaglandins; **S**teroidogenesis; Ewes; Partuririton; Aglepristone; Dexamethasone

INTRODUCTION

Physiological progesterone withdrawal and concomitant increase of estrogen production during parturitions are mostly associated with expressions of several enzymes required in placental steroidogenesis. The mechanism of sheep parturition is initiated by the increase of fetal cortisol concerning maturation of hypothalamic–pituitary–adrenal (HPA) axis, which induces upregulation of $PTGS_2/COX_2$ and CYP17 genes, causing an enhanced synthesis of PGE_2 (Challis *et al.,* 2001; Braun *et al.,* 2013; Schuler *et al.,* 2018). CYP17 expression catalyzes 17α-hydroxylation and 17,20 lyase reactions to convert pregnenolone to 17α-hydroxypregnenolone and dehydroepiandrosterone (DHEA) at the end of pregnancy. An increase in estrogen production results from aromatization of DHEA into estrogens by a key enzyme, P450arom, which is encoded as CYP19 (Flint *et al*., 1975; Mason *et al.,* 1989; Vanselow et *al.,* 2008). Consequently, a decrease in placental progesterone output is suggested to be associated with the synthesis of estrogens via the Δ5-pathway of steroidogenesis, rather than direct conversion of progesterone to estrogens (Mason *et al.,* 1989).

During alterations in placental steroidogenesis around parturition, high levels of progesterone give place to high concentrations of estrogens, leading to the release of uterotonic prostaglandins and upregulation of contraction-associated proteins in the myometrium (Challis, 1971; Tsang, 1974; Gyomorey *et al.,* 2000; Whittle *et al.,* 2000; Challis and Lye, 1994; Lye, 1994). $PGF_{2\alpha}$ and PGE_2 are considered to be key mediators of myometrial contractions and differential expression of prostaglandin enzymes in uterus occurred at a time course similar to that of rise in estrogen synthesis (Lye, 1994; Palliser *et al.,* 2006). Myometrial contractility is mediated by these important hormones acting on prostaglandin E (EP) and prostaglandin F (FP) receptors. FP receptors have been suggested as strong stimulators, whereas EP receptors could be active either in contraction or relaxation, depending on EP subtypes (EP1-4) (Palliser *et al.,* 2005).

Pregnancy ketosis or pregnancy toxemia is a disruption of glucose homeostasis, characterized by hypoglycemia and hyperketonaemia, which adversely affects ewes' brain and nervous system function during the last month of pregnancy (Rook, 2000; Zamir and Rozov, 2009; McClymont and Setchell, 1956). Traditional therapies mostly focus on correcting clinical findings associated with glucose demands (Boileau, 2008). Further to administering oral propylene glycol and intravenous dextrose solutions, corticosteroids are added to the protocols to assist gluconeogenesis, increase appetite and induce parturition (Rook, 2000; Smith and Sherman, 1994; Radostits *et al.,* 2007).

A progesterone receptor blocker, aglepristone (RU46534) has been tested in treatments of various progesterone-dependent physiological or pathologic conditions in small and large animals (Guil-Luna *et al.,* 2011; Fontbonne *et al*., 2009; Shenavai *et al.,* 2010; Shenevai *et al*., 2012; Özalp *et al*., 2008; Batista *et al*., 2011; Özalp *et al*., 2017). As an abortifacient it could be safely used in small animal practices, however, its successful effects have been revealed in parturition induction in large animals (Shenavai *et al.,* 2010; Shenevai *et al.,* 2012; Batista *et al*., 2011; Özalp *et al*., 2017). Clinical efficiency of aglepristone in parturition induction in goats, ewes, and cows is proved, even in pathologic conditions in ewes. Our studies have suggested that aglepristone could be a useful agent in parturition induction in ewes with pregnancy toxemia and healthy ones. It precisely controls lambing time without any side effects in either mothers or lambs (Özalp *et al.,* 2018).

Dexamethasone, a synthetic pregnane corticosteroid, has been thoroughly tested in parturition induction in ewes with pregnancy toxemia (Kastelic *et al.,* 1996; Hunt, 1976a; Hunt, 1976b). A high incidence of dystocia in stage II parturition has been frequently reported in ewes with pregnancy toxemia, whether parturition induction was performed. Previous studies

and our clinical observations of the last ten years have confirmed severe dystocia with incomplete cervical dilatation (ringwomb), especially after dexamethasone administration in pregnancy toxemia (Özalp *et al.,* 2018). Liggins suggested that the reason for large doses of dexamethasone that fail to cause parturition could be associated with the influence of possible indirect action of corticosteroids on placental progesterone (Liggins *et al.,* 1971). The changed expression of steroidogenic enzymes and concentrations of circulating hormones had been discussed in undernourished or overnourished ewes, as balanced nutrition had great importance in endocrinological environment during pregnancy and parturition (Bloomfield *et al.,* 2004; Reynolds *et al.,* 2010; Long *et al.,* 2013). However, limited information about the alterations in placental steroidogenesis and prostaglandins ratio in ewes with pregnancy toxemia is available. Although dexamethasone and aglepristone could be successfully used as parturition inducers in bitches, queens, cows, goats and ewes, still their parturition induction role in ewes with pregnancy toxemia is unclear. Hence, this study aimed to examine possible alterations of the normal pattern of a series of enzymes and receptors in placental steroidogenesis and prostaglandins according to the induction protocol and/or the toxemia status of the pregnant ewe. The abbreviations and molecular names of target genes examined in this study are listed below in Table 1.

Table 1: List of the abbreviations and molecular names of target genes

Abbreviations	Molecule Name
FP	prostaglandin F
PGFM	13,14-dihydro-15-keto-prostaglandin F2α
$PTGS_2$/COX_2	Prostaglandin-endoperoxide synthase 2/cyclooxygenase 2
PGFS	PGF2α synthase
PTGES	PTGE2 synthase
FP	PGF2α receptor
$PTGER_1$/EP1	PGE2-receptor
$PTGER_2$/EP2	PGE2-receptor
$PTGER_3$/EP3	PGE2-receptors
$PTGER_4$/EP4	PGE2-receptors
CYP11A	cholesterol side-chain cleavage enzyme
CYP17	17-α-hydroxylase/17,20 lyase
3βHSD	3β-hydroxysteroid dehydrogenase/ Δ5-4 isomerase
CYP19	P450arom
STARpr	steroidogenic acute regulatory protein

MATERIAL AND METHOD

1. Animals and Experimental Design

Twenty-six healthy crossbreeds (Kivircik X Chios) ewes aged four to five years old, were subjected to estrus synchronization by inserting intravaginal sponges containing 30 mg of cronolone fluorogestone acetate (Chronogest CR, Intervet, Turkey) for 11 days and received 400 IU pregnant mare serum gonadotrophin (Chronogest/PMSG, Intervet, Turkey) IM when the sponge was withdrawn (Özalp *et al.,* 2018). The ewes were exposed to rams on days 12-14, and the breeding date was recorded. The animals presented homogeneous body condition score above 2.5. They were housed in a sheep farm in Görükle region in Bursa, Turkey, 40° N and 28° E at an altitude of 128 m above sea level. Ethics committee approval was received for this study from the ethics committee of Bursa Uludag University (2015-03/11) (Özalp *et al.,* 2018). The pregnancy confirmation and determination of the number of fetuses were carried out by ultrasonography on days 35-40.

All ewes *(n=26)* were fed well until day 125 of pregnancy (0.45 kg whole corn, 1.2 kg alfalfa hay, and barley straw/ewe; (equivalent to 82% of daily needs, 3.1 Mcal ME); and after that day, except for the negative control group *(n=4),* each ewe carrying one fetus and two fetuses were fed with 0.50 kg of reduced energy concentrate feed plus 0.50 kg of alfalfa hay and 0.60 kg and 0.50 kg, respectively *(n=22)* (Barbagianni *et al.,* 2015). The animals were observed until typical clinical symptoms of pregnancy toxemia as described before (Ranaweera *et al.,* 1979). Severe ketonuria from urine samples and increased NEFA and β-HBA concentrations accompanied the disease and all ewes developed clinical pregnancy toxemia (Özalp *et al.,* 2018). The clinical findings and the diagnosis of pregnancy toxemia by laboratory tests had been explained in our previous paper (Özalp *et al.,* 2018).

The ewes with pregnancy toxemia were randomly assigned into three groups and they received the treatment protocol assigned to each group. Clinical and laboratory results initiated the treatment protocol and parturition inductions right after the pregnancy toxemia diagnosis. Group Aglepristone (AG/ *n=9*; 56,3±2,29 kg); animals received 30% dextrose solution (Polifleks®, Polifarma Medicine Ind. and Trade. Inc., Tekirdağ) intravenously (dose: 100-200 mL/animal) and 100 ml oral administration of propylene glycol twice daily, until the end of delivery of fetuses and placentas. Parturition was induced by

aglepristone (Alizin, Virbac, Germany) twice with subcutaneous injection of 10 mg/kg at a 24 h interval. The treatment began on day 141±1,3 of gestation. Group Dexamethasone (DEX/ *n=9;* 60,3±4,94 kg); ewes received uniform treatment protocol for energy demand as in Group AG. The lambs of two ewes in group AG were extracted by traction because of parturition pathology. Fifteen mg, of single dexamethasone (Devamed, Topkim, Turkey) was subcutaneously applied to this group to terminate pregnancy. Due to incomplete cervical dilatation, the lambs in group DEX were delivered by cesarean section in 8 ewes. The treatment began on day 141,6±2,11 of gestation. Group Pregnancy Toxemia (PT/ *n*=4; 56,2±1,50 kg); ewes were only treated with 30% dextrose solution and propylene glycol, and the lambs were removed by cesarean section. The operations were performed on day 146,2±2,28 of gestation. Control Group (CG/ *n*=4; 56,7±3,40 kg); animals received neither undernutrition program, parturition induction treatment nor C-section and they performed normal birth (Özalp *et al.,* 2018).

2. Plasma sample collection

Blood samples (2mL) were drawn into EDTA vacuum tubes and collected from vena jugularis of all ewes using 18 g needles. The sampling was initiated before the administration of aglepristone or dexamethasone and thereafter until the end of placental delivery samples were collected every 12 hours. Tubes were immediately centrifugated (3,000 × g, 15 min) and plasma samples were frozen at −80°C until analyses were performed. The ELISA reader determined hormone concentration measurements (BioTek Instruments, USA).

The PGFM, cortisol, PGE_2 and estrone sulfate concentrations in the maternal samples were determined using commercial ovine ELISA kits [PGFM, 15-keto-13, 14-dihydro-prostaglandin $F_{2\alpha}$, (MyBioSource, MBS7219926), cortisol (Abnova, KA2317), PGE_2 (Cusabio, CSB-EQ027602SH), estrone sulfate (Abnova, KA2319)]. The samples for ELISA were diluted 2-fold and 100-fold with assay buffer for PGFM and estrone sulfate, respectively.

3. Tissue collection and mRNA isolation

Immediately after the delivery of the last lamb, either by cesarian section or normal parturition, three to five placentomes were removed by operation. The placentomes were collected by the cesarian section in all ewes in 30 minutes. For total RNA extraction placentome capsule, caruncle, and cotyledon were carefully dissected, snap-frozen in liquid nitrogen and stored at -80°C. The different anatomical structures were analyzed separately to identify the mechanisms of placental steroidogenesis and prostaglandin production. Generally, parturition is controlled by regulations of tissue-specific arrays of steroidogenic enzymes in ruminants. Fetal cotyledons are responsible for placental steroid production which form multiple discrete sites of placentation with maternal caruncular tissue (Schuler *et al.,* 2006; Hoffmann *et al.,* 1979; Conley and Ford, 1987; Whittle *et al.,* 2001).

Deep-frozen tissue samples were quickly pulverized using pestle and mortar under liquid nitrogen. According to the manufacturer's instructions, total RNA was isolated using the innuPREP RNA Mini Kit (*Analytik Jena* AG, Jena, Germany). On an ethidium bromide- stained 1% agarose gel, RNA integrity was tested by the presence of intact bands of 12s and 28s. mRNA purity and quantity were determined by optical density (OD) measurement (NanoDrop 2000c, Thermo Scientific, USA). All samples' OD 260/280 ratio was between 1.8 and 2.0.

4. Real-Time PCR measurements

Fifteen microliters of total RNA (1.0 μg) in 20 μL final volume were then reverse transcribed with 4 μl 5xiScriptReactionMix and 1μl iScript Reverse Transcriptase (iScript™ cDNA Synthesis Kit, Bio-Rad) Real-time PCR was performed in a LightCycler 480II thermocycler (Roche) using the SYBR Green method (LightCycler® 480 SYBR Green I Master, Roche). According to the MIQE (minimal information for publication of quantitative real-time PCR experiments) guideline, all qPCR analyses and measurements were carried out. The qPCR efficiency for each set was >99%, as determined by the equation E= 10(−1/slope)−1. Each sample was measured in triplicate. Primer pairs for the target genes CYP11A, *CYP17, CYP19*, 3βHSD, STAR protein, $PTGS_2$/COX_2, PGFS, PTGES, FP, PTGER1/EP1, PTGER2/EP2, PTGER3/EP3 and PTGER4/EP4 and the reference gene β-actin were adopted from previous publications (Table 2) or designed using NCBI - Ensemble and purchased from Macrogen Inc. (Korea). Amplification conditions were the same for the target and the reference genes: initial denaturation for 5 min at 95 °C followed by 45 cycles at 95 °C for 10 s, 57 °C for 10 s and 72 °C for 10s. Melting curve analysis was used for each primer pair to confirm the gene-specific peak and the lack of primer dimer. E-method evaluated re-

Table 2: Sequences for primers

Target Gene	Forward Primer Sequence (5'->3')	Reverse Primer Sequence (5'->3')	Genbank accession no.
$PTGS_2$/COX_2	CTTCAAGGGAGTCTGGAACATT	AAGTTGGCGGACTCTCAATC	NM_001009432.1
PGFS	CCCTTCGCTATCAGGTACAAC	GGAGTCAGTTCAAAGTCAAACAC	XM_012188608.2
PTGES	CCTCCAGTATTGCCGGAAC	GTAGACAAAGCCCAGGAACA	XM_012118326.2
FP	TGCATTTGTTGGAGTCCATTTC	TCCATGTTGCCATTCGAAGA	XM_012162853.2
$PTGER_1$/EP1	CACACGACGTGGAGATGG	ACCACCACCAGCAAAGG	XM_015095782.1
$PTGER_2$/EP2	GAGGAGACGGACCATCTTATTC	GCTTGGAGGTCCCACTTT	NM_001278560.1
$PTGER_3$/EP3	GGACTAACTCCCGGCAAAAT	CTGTGTATGTCTTGCAGTGC	XM_012173631.2
$PTGER_4$/EP4	GGAGACGACTTTCTACACACTGGT	TGATGCTGAGCCCCGACA	XM_015101313.1
CYP11A	ACCGTCTGTTCAGAACCAAG	TCTGTCTCAGATCCTGGTAGAA	NM_001093789.1
CYP17	GGATGGCAACCTGAAGTTAGAG	AGGCTCGGACAGATCTATGG	XM_012102863.2
3βHSD	AATCCGGGTGCTAGACAAAG	CTCATCCAGAATGTCTCCTTCC	XM_012183658.1
CYP19	AGCTGTTCGACCTTTCTTTACA	TGTCCAGATGCTTGGTGATG	NM_001123000.1
STARpr	CGACCAAGAGCTTGCCTATATC	CACGTCAGGGATCACTTTACTC	NM_001009243.1
Actin β*	TCTTCCAGCCTTCCTTCCT	TAGAGGTCCTTGCGGATGT	NM_001009784.2

sults. To determine the relative expression of genes Ct value was normalized by β-Actin. The values in the negative control group (CG) were compared with AG, DEX and PT groups and fold change values were calculated.

5. Statistical Analysis

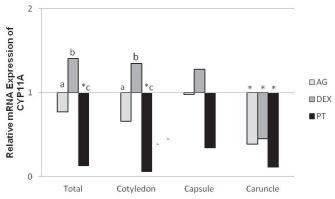
The statistical analysis of hormone concentrations was analyzed using Kruskal-Wallis variance analysis ($P<0.05$). The Bonferroni Mann Whitney U test as the post hoc in multiple group comparisons was used for the significant parameters in the Kruskal Wallis test. Results were expressed as the median for each category.

The Real-Time PCR data were subjected to Kruskal-Wallis using $2^{-\Delta\Delta Ct}$ values to determine statistically significant differences in all gene expressions. Expression values in AG, DEX, and PT groups were checked against the values in the control group (CG). Gene expression of CG was set at 1 and related to negative the control group. All statistical analysis was performed with SPSS software (SPSS for Windows. Standard version release 11,5. Copyright SPSS Inc., 2002, the USA).

RESULTS

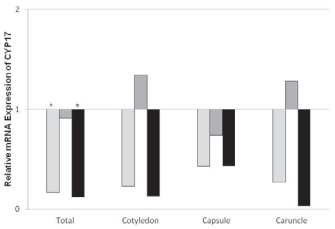
1. Expression of steroidogenic enzymes, STAR protein in placentomes

The lower mRNA expression of CYP11A, CYP17, 3βHSD, CYP19 and STAR protein was detected in all tissue types in PT group. CYP11A mRNA expression was statistically different in caruncle between all groups, in total placentome and cotyledon in PT group compared to CG ($P<0.005$). CYP17 mRNA expression was greater only in cotyledon and caruncle of DEX group, however, the expression of this gene was statistically different only in total placentome in AG and DEX groups compared to CG ($P<0.05$). STAR protein mRNA expression was also lower in all groups except for total and capsule of DEX. The difference was statistically found in total placentome between AG-PT groups compared to CG. No significance could be detected in 3βHSD and CYP19 expressions between groups and tissue types ($P>0.05$). Relative expression of target gene-specific mRNA results of STAR protein and steroidogenic enzymes between groups and significant error probabilities of expressions were presented in Figures 1,2, 3, and 4.



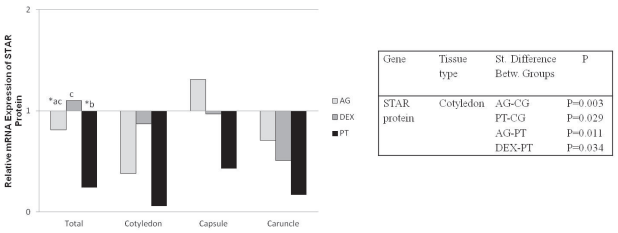
Gene	Tissue type	St. Difference Betw. Groups	P
CYP11A	Cotyledon	PT-CG	P=0.029
		AG-DEX	P=0.040
		AG-PT	P=0.003
		DEX-PT	P=0.020
CYP11A	Total	PT-CG	P=0.029
		AG-PT	P=0.020
		DEX-PT	P=0.050
CYP11A	Caruncle	AG-CG	P=0.003
		DEX-CG	P=0.034
		PT-CG	P=0.029

Figure 1: qPCR of CYP11A in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups



Gene	Tissue type	St. Difference Betw. Groups	P
CYP17	Total	AG-CG	P=0.003
		PT-CG	P=0.029

Figure 2: qPCR of CYP17 in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups



Gene	Tissue type	St. Difference Betw. Groups	P
STAR protein	Cotyledon	AG-CG	P=0.003
		PT-CG	P=0.029
		AG-PT	P=0.011
		DEX-PT	P=0.034

Figure 3: qPCR of STAR protein in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups

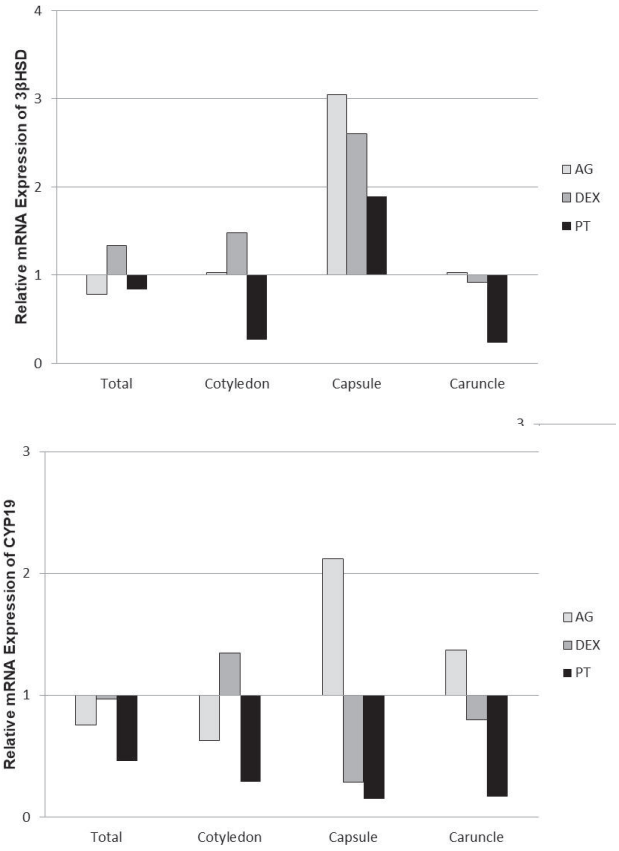
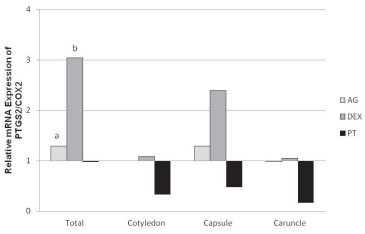


Figure 4: qPCR of 3βHSD-CYP19 in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups

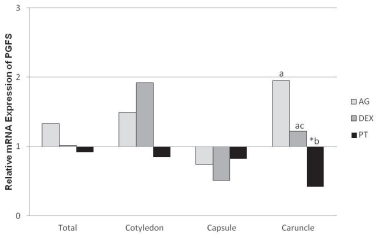
2. Expression of PGFS, $PTGS_2/COX_2$ and FP in placentomes

$PTGS_2/COX_2$ mRNA expression was lower in PT group in cotyledon, capsule and caruncle. No statistical difference was detected in all tissue and groups compared to CG, whereas greater expression of $PTGS_2/COX_2$ was different between groups AG and DEX ($P<0.05$). The PGFS mRNA expression was different only in caruncle between groups PT-CG, AG-PT DEX-PT statistically ($P<0.05$). No significance could be detected in FP expression between groups and tissue types ($P>0.05$). Relative expression of target gene-specific mRNA results of PGFS, $PTGS_2/COX_2$ and FP between groups and significant error probabilities of expressions were presented in Figures 5, 6, 7.



Gene	Tissue type	St. Difference Betw. Groups	P
PTGS2/ COX2	Total	AG-DEX	P=0.038

Figure 5: qPCR of PTGS2/COX2 in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups



Gene	Tissue type	St. Difference Betw. Groups	P
PGFS	Caruncle	PT-CG	P=0.029
		AG-PT	P=0.003
		DEX-PT	P=0.006

Figure 6: qPCR of PGFS in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups

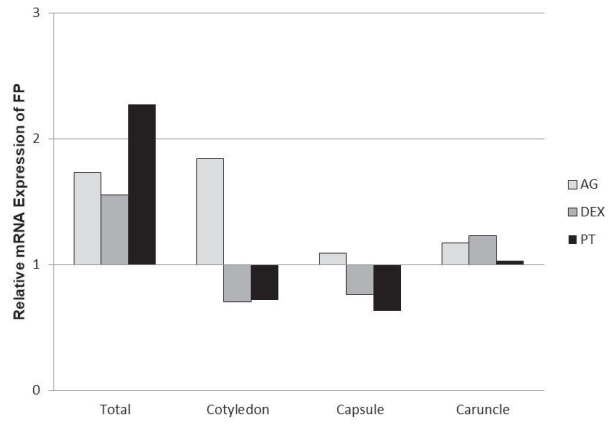


Figure 7: qPCR of FP in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups

3. Expression of PTGES and $PTGER_1$/EP1, $PTGER_2$/EP2, $PTGER_3$/EP3, $PTGER_4$/EP4 in placentomes

A significant could be observed in $PTGER_3$/EP3 expression in caruncle of DEX and AG compared to CG ($P<0.05$); however PTGES, $PTGER_1$/EP1, $PTGER_2$/EP2 and $PTGER_4$/EP4, expressions were not statistically different among groups ($P>0,05$). Relative expression of target gene-specific mRNA results of PTGES, $PTGER_1$/EP1, $PTGER_2$/EP2, $PTGER_3$/EP3 and $PTGER_4$/EP4, between groups and signifi-

cant error probabilities of expressions, were presented in Figures 8, 9 and 10.

Then, gene expressions, responsible for uterine extractions were described as downregulated or upregulated, according to tissue types and groups in Figure 16.

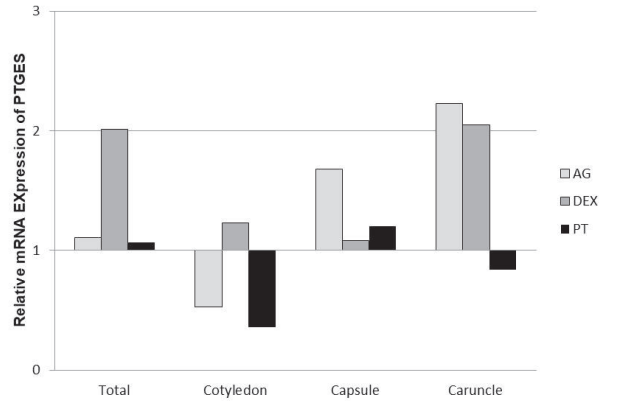


Figure 8: qPCR of PTGES in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups

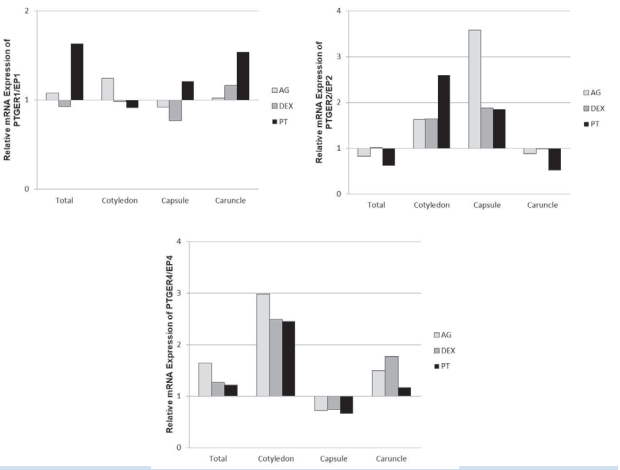
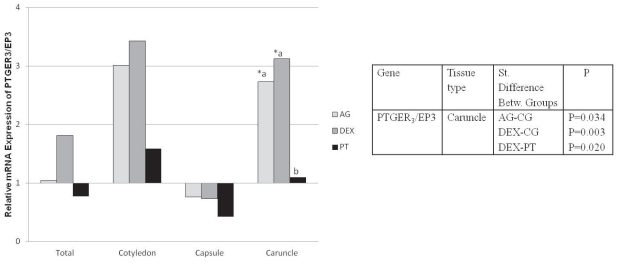


Figure 9: qPCR of EP1-EP2-EP4 in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups

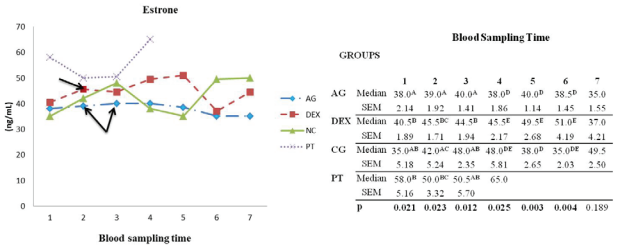


Gene	Tissue type	St. Difference Betw. Groups	P
PTGER$_3$/EP3	Caruncle	AG-CG	P=0.034
		DEX-CG	P=0.003
		DEX-PT	P=0.020

Figure 10: qPCR of EP3 in total, cotyledon, capsule and caruncle placentome in AG, DEX and PT groups

4. Hormone Concentrations

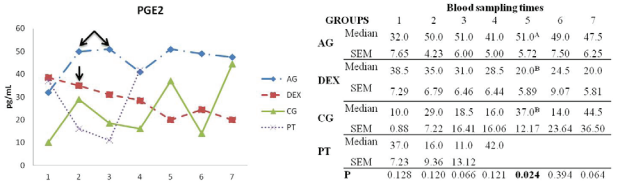
Estrone sulfate (ng/mL) and progesterone (ng/mL) concentrations were statistically different in the first three sampling times between groups (P<0,012-P<0,023; P<0,002-P<0,007, resp) (Figure 11,15). On the other hand, the measurements of 4., 5.and 6. sampling of estrone sulfate concentration were also different between AG-DEX and CG groups (P<0,003-P<0,025) (Figure 11). The statistical difference was detected in PGE_2 (pg/mL) and PGFM (pg/mL) concentrations between AG-DEX and CG in 5.and 1-3.sampling time, respectively (P<0,024; P<0,017, P<0,034, resp.) (Figure 12,13). No difference was observed in cortisol (ng/mL) concentrations between groups (P>0,05) (Figure 14).



Blood Sampling Time

GROUPS		1	2	3	4	5	6	7
AG	Median	38.0^{A}	39.0^{A}	40.0^{A}	38.0^{D}	40.0^{D}	38.5^{D}	35.0
	SEM	2.14	1.92	1.41	1.86	1.14	1.45	1.55
DEX	Median	40.5^{B}	45.5^{BC}	44.5^{B}	45.5^{E}	49.5^{E}	51.0^{E}	37.0
	SEM	1.89	1.71	1.94	2.17	2.68	4.19	4.21
CG	Median	35.0^{AB}	42.0^{AC}	48.0^{AB}	48.0^{DE}	38.0^{D}	35.0^{DE}	49.5
	SEM	5.18	5.24	2.35	5.81	2.65	2.03	2.50
PT	Median	58.0^{B}	50.0^{BC}	50.5^{AB}	65.0			
	SEM	5.16	3.32	5.70				
p		**0.021**	**0.023**	**0.012**	**0.025**	**0.003**	**0.004**	0.189

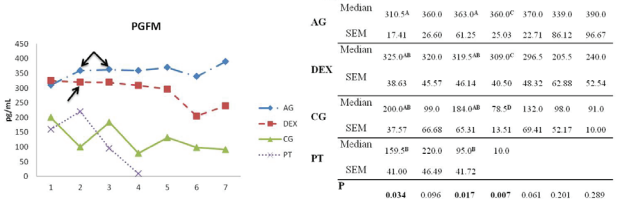
Figure 11: Estrone sulfate concentrations in AG-DEX-PT and CG groups



Blood sampling times

GROUPS		1	2	3	4	5	6	7
AG	Median	32.0	50.0	51.0	41.0	51.0^{A}	49.0	47.5
	SEM	7.65	4.23	6.00	5.00	5.72	7.50	6.25
DEX	Median	38.5	35.0	31.0	28.5	20.0^{B}	24.5	20.0
	SEM	7.29	6.79	6.46	6.44	5.89	9.07	5.81
CG	Median	10.0	29.0	18.5	16.0	37.0^{B}	14.0	44.5
	SEM	0.88	7.22	16.41	16.06	12.17	23.64	36.50
PT	Median	37.0	16.0	11.0	42.0			
	SEM	7.23	9.36	13.12				
P		0.128	0.120	0.066	0.121	**0.024**	0.394	0.064

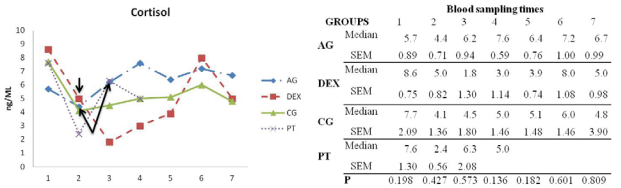
Figure 12: PGE2 concentrations in AG-DEX-PT and CG groups



Blood sampling times

GROUPS		1	2	3	4	5	6	7
AG	Median	310.5^{A}	360.0	363.0^{A}	360.0^{C}	370.0	339.0	390.0
	SEM	17.41	26.60	61.25	25.03	22.71	86.12	96.67
DEX	Median	325.0^{AB}	320.0	519.5^{AB}	309.0^{C}	296.5	205.5	240.0
	SEM	38.63	45.57	46.14	40.50	48.32	62.88	52.54
CG	Median	200.0^{AB}	99.0	184.0^{AB}	78.5^{D}	132.0	98.0	91.0
	SEM	37.57	66.68	65.31	13.51	69.41	52.17	10.00
PT	Median	159.5^{B}	220.0	95.0^{B}	10.0			
	SEM	41.00	46.49	41.72				
P		**0.034**	0.096	**0.017**	**0.007**	0.061	0.201	0.289

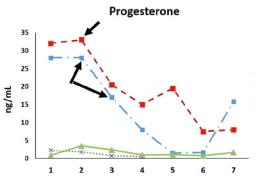
Figure 13: PGFM concentrations in AG-DEX-PT and CG groups



Blood sampling times

GROUPS		1	2	3	4	5	6	7
AG	Median	5.7	4.4	6.2	7.6	6.4	7.2	6.7
	SEM	0.89	0.71	0.94	0.59	0.76	1.00	0.99
DEX	Median	8.6	5.0	1.8	3.0	3.9	8.0	5.0
	SEM	0.75	0.82	1.30	1.14	0.74	1.08	0.98
CG	Median	7.7	4.1	4.5	5.0	5.1	6.0	4.8
	SEM	2.09	1.36	1.80	1.46	1.48	1.46	3.90
PT	Median	7.6	2.4	6.3	5.0			
	SEM	1.30	0.56	2.08				
P		0.198	0.427	0.573	0.136	0.182	0.601	0.809

Figure 14: Cortisol concentrations in AG-DEX-PT and CG groups



GROUPS		Blood sampling times. 1	2	3	4	5	6	7
AG	Median	28,0^{AB}	28,0^{AB}	17,0^{A}	8,0	1,5	1,7	15,8
	SEM	5,14	4,26	4,71	4,84	3,85	4,43	7,78
DEX	Median	32,0^{B}	33,0^{B}	20,5^{A}	15,0	19,5	7,5	8,0
	SEM	1,67	3,50	4,19	3,32	4,50	4,26	3,81
CG	Median	0,9^{A}	3,5^{AC}	2,4^{AB}	1,0	1,1	0,8	1,7
	SEM	0,10	0,38	0,75	0,18	0,74	0,40	0,25
PT	Median	2,3^{A}	1,8^{C}	0,8^{B}	0,6			
	SEM	5,77	0,41	0,15				
P		**0,007**	**0,002**	**0,004**	0,057	0,192	0,320	0,53

Figure 15: Progesterone concentrations in AG-DEX-PT and CG groups

DISCUSSION

The present study suggests that the endocrinologic pathway controlling parturition is not in the same direction in ewes with pregnancy toxemia, observed in healthy ones. Maturation of fetal HPA axis, enzymatic changes in trophoblast, leading endocrinologic alterations in placentome and activation of myometrium are important steps triggering physiological initiation of parturition in ewes (Lye, 1994; Whittle *et al.,* 2001). Recent studies have revealed that the rise in fetal cortisol occurred with a time course similar to that of the rise in fetal PGE_2, which could increase intrauterine prostaglandin production at the onset of parturition (Gyomorey *et al.,* 2000; Whittle *et al.,* 2000; Challis *et al.,* 1997). Although fetal cortisol, ACTH and PGE_2 concentrations were not measured in our study, high concentrations of PGE_2, cortisol, estrone sulfate and low PGFM concentration were detected in maternal plasma in PT ewes. Furthermore, downregulation of STAR protein and placental enzymes, CYP11A, 3βHSD, CYP17, CYP19, as well as $PTGS_2$/COX_2 expression in all parts of placentome have been detected in ewes with pregnancy toxemia in our study. Notwithstanding downregulation of placental enzymes regulating the initiation of parturition in PT group, the existence of different endocrinologic pathways controlling parturition in pregnancy toxemia, could be suggested. The high concentration of estrone sulfate could not be due to placental alterations, as low expressions of $PTGS_2$/COX_2, CYP17 and CYP19 have been measured in this group. Low concentration of progesterone due to insufficient nutrition might directly trigger endometrial prostaglandin production in ewes, as it's suggested in cows (Shenavai *et al.,* 2012). Disorders in progesteron and estrogen mechanisms are related to nutritional restriction and insulin resistance in women. This could be also suggested as a causative factor in ewes with pregnancy toxemia (Barros *et al.,* 2008; Root-Bernstein *et al.,* 2014; Duehlmeier *et al.,* 2013). About hypoglycemia, high glucocorticoid concentration could be the consequence of increased adrenal output or poor conjugation of damaged liver (McClymont and Setchell, 1956; McClymont and Setchell, 1995; Ford et *al.,* 1990). On the other hand, hypoglycemic condition induces a natural physiological response by the elevation of endogenous steroid levels.

Stress factors and insufficient nutrition causing high production of cortisol and ACTH in both mother and fetus respectively, but not fetal PGE_2, has probably evoked a parturition process in pregnancy toxemia. High ACTH concentrations in lambs, which of all not had elevated cortisol, leading to accelerated HPA maturation in undernutrition had been reported (Bloomfield *et al.,* 2003). The absence of $PTGS_2$/COX_2 and CYP17 upregulation in trophoblast cells could reveal a different route of parturition associated with non-contributing placental enzymes in pregnancy toxemia. However, upregulation of FP, PTGES and its receptors due to insufficient regulation of placental enzymes is still unclear. Spontaneous parturition in pregnancy toxemia could occur due to immune and inflammatory processes. Although non-infectious onset of parturition is pronounced in pregnancy toxemia, by-products of fat metabolism, ketone bodies, could act as a foreign body and activate PGE_2, an important lipid mediator during inflammation (Lewis *et al.,* 1978). The lowest PGFM concentration and the downregulation of PGFS gene in placentome could support this hypothesis in pregnancy toxemia in this study. Decreased PGFM level in this study could also clarify the reason for dystocia during pregnancy toxemia, associated with suppressive effects of progesterone levels via non-genomic actions on myometrium (Shenavai *et al.,* 2012; Stormshak and Bishop, 2008). Aglepristone, a progesterone receptor antagonist, could control lambing time without any side effects in mothers and newborns in ewes with pregnancy toxemia (Özalp *et al.,* 2018). Although aglepristone seemed more effective in parturition induction clinically in group AG, placental steroid enzyme regulation of group AG was observed as similar to group PT. Interestingly, no upregulation of these important enzymes in cotyledon, except for CYP19, could be detected in AG ewes. Downregulation of cotyledonary CYP17 and $PTGS_2$/COX_2 expressions, key enzymes in the signal cascade leading to the onset of parturition, was also unexpected in this study. Reduced expression of STAR protein, CYP11A and 3βHSD, CYP17 rates could be related to impaired progesterone production and insulin mechanism, as it was suggested in the etiopathogenesis of polycystic ovary syndrome in women (Huang *et al.,* 2016; Nestler and Jakubowicz, 1996; Qin and Rosenfield,

1998). Therefore, it remains possible that unchanged placental functions have intensive relationship with pregnancy ketosis because, the placental enzyme regulations were similar to group PT. Functional progesterone withdrawal associated with progesterone receptor blockage could probably stimulate prostaglandin production, as it's suggested in cows (Shenavai *et al.,* 2012).

The increase of fetal cortisol, PGE_2, ACTH and maternal PGFM concentrations had been explained after RU486 administration in ewes in late gestation (McKeown *et al.,* 1997). Based on the observations of the present study, we suggest that aglepristone application might promote the production of fetal hormones and maternal PGE_2 and PGFM. Since PGFS, PTGES and their receptors were upregulated in caruncle, direct contribution to endometrial prostaglandin production by aglepristone may be argued. From another point of view, an existing placental steroid production could be masked by metabolism or aglepristone could only contribute to immune and inflammatory processes leading to parturition, by stimuli of fetal signal to the endometrial response.

Due to mimicking fetal cortisol, dexamethasone applications are recommended to induce parturition and gluconeogenesis in traditional treatment protocols for decades in pregnancy toxemia (Boland *et al.,* 1982; Harman and Slyter, 1980). Nevertheless, dystocia and/or insufficient cervical dilatation are significantly higher diagnosed in pregnancy toxemia, with or without dexamethasone. Recently, we have shown that the ewes treated with aglepristone have only poor widening of birth canal, so the lambs could be extracted easily, however, the ewes treated with dexamethasone required cesarean section due to incomplete cervical dilatation (Özalp *et al.,* 2017; Özalp *et al.,* 2018). Placental enzymes and prostaglandin regulations had similarities with normal parturition even though having such pregnancy pathologies in DEX group. Dexamethasone stimulation provides clear evidence that $PTGS_2/COX_2$ and CYP17 are being upregulated in cotyledon, leading to PGE_2 and PGFM production, which is consistent with the results of Whittle *et al.* (2000). The upregulation of the enzymes that play role in biochemical pathways of steroid synthesis in our study, in accordance with previous studies, reporting specific regulation changes of CYP11A, 1.5-2 fold increase of CYP19 and 200-fold increase CYP17 activity after dexamethasone application. Possible molecular regulation of CYP17 was speculated to be controlled by glucocorticosteroid receptor binding region corresponding to the 5'-flanking site (France *et al.,* 1987; France *et al.,* 1998).

The activities of PTGES and PGFS are predominant pathways for PGE_2 and $PGF_{2\alpha}$ formation, respectively (Palliser *et al.,* 2004; Watanabe *et al.,* 1985). Elevated cotyledonary PGFS expression and increased PTGS2/COX2 likely lead to the increase of caruncular PTGES and subsequent plasma PGE2 in the DEX group. However unchanged PTGES and decreased PGFM and FP expressions were proposed in myometrium and amnion of ewe after dexamethasone by Palliser *et al.* (2004). Tissue-specific roles of prostaglandins appear to differ markedly between the health status of ewes during parturition, as the main route of endocrinology is still unclear in pregnancy toxemia.

Specific receptors of prostaglandins exert a shift from uterine quiescence to contractility, which are EP1, EP2, EP3, EP4 and FP (Negishi *et al.,* 1995; Challis *et al.,* 2000). Contractile (EP1, EP3, FP) and relaxatory (EP2, EP4) receptor isoforms have the highest affinity for PGE_2 and $PGF_{2\alpha}$, which play central a role to regulate contractility during labor (Challis and Lye, 1994; Ma *et al.,* 1999). Regional variations and differential distribution of these receptors responding to prostaglandins in myometrium, endometrium and placentome have been revealed (Palliser *et al.,* 2005; Ma *et al.,* 1999; Smith *et al.,* 1998). The evidence of the largest capacity of prostaglandin production in placentomes could be a new debate in controlling myometrial activity, as prostaglandin receptors had regulation patterns in cotyledon and caruncle in this study (Palliser *et al.,* 2004; McLaren *et al.,* 1996). The action of dexamethasone on transcriptional level of prostaglandin receptors was observed similar to group PT. Interestingly, FP, EP1 and EP3 that stimulate excitatory response were upregulated in caruncle. At the same effects and EP4 affects the inhibitory response, was upregulated in cotyledon in DEX and PT groups in our study. On the other hand, the different regulation pattern of cotyledonary FP and EP1 was observed in AG (Figure 16). The clinical efficiency of aglepristone having less parturition pathology in pregnancy toxemia could associate with cotyledonary expression of prostaglandin receptors. Myometrial/endometrial EP3 and FP expressions controlling myometrial activity have been presented in previous studies (Palliser *et al.,* 2006; Ma *et al.,* 1999; Konopka *et al.,* 2015). Nevertheless undetect-

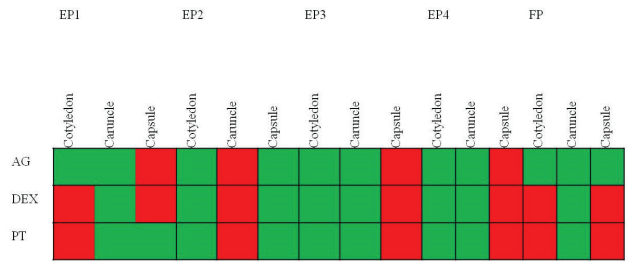


Figure16: Downregulated/upregulated gene expressions of prostaglandin receptors, responsible for uterine contractions during parturition

able or extremely low level of EP1 has not been taken into consideration either in spontaneous or dexamethasone-induced parturition (Ma *et al.,* 1999; Konopka *et al.,* 2015). Significant upregulation of cotyledonary FP, EP1 and EP3 expressions in AG ewes could be the reason for decreased pathologies during parturition. On the other hand, it would be possible to defend the high incidence of ringwomb in PT and DEX groups with the arguments of downregulation of EP1 and FP expressions in these groups. The counter-expression of cervical EP genes concerning myometrium/endometrium could refer to the pathogenesis of insufficient cervical dilatation. Tissue-specific changes and differential regulation of EP receptors had been demonstrated in women during parturition (Konopka *et al.,* 2015). Nevertheless, further studies, including cervical EP expressions, are needed to clarify the pathogenesis of insufficient cervical dilatation in pregnancy toxemia in ewes. On the other hand, new information about other contraction-associated proteins is needed to introduce the exact mechanism of parturition pathologies in pregnancy toxemia.

CONCLUSION

In summary, the expressional profile of tissue-specific counter-expressions of EP and FP genes had shown the probable mechanism of insufficient cervical dilatation, observed in ewes with pregnancy toxemia and after dexamethasone applications. Hormonal imbalance in pregnancy toxemia and large doses of dexamethasone leading to ineffective progesterone action have been suggested for this pathology. As was supported due to the expression pattern of placental enzymes and prostaglandins, the endocrinologic main route of spontaneous labor was not observed in ewes with pregnancy toxemia. Treatment protocols causing hormonal alterations had indicated different expression patterns in the parturition process in ewes with pregnancy toxemia. The ineffective uterine contractions were speculated to be evidence of differences in expressions of PG production (Özalp *et al.,* 2018).

ACKNOWLEDGMENTS

This study was supported financially by Technical Research Council of Turkey (TUBITAK/TOVAG 214O673). The authors of this paper are members of COST (European Cooperation in Science and Technology) Action BM1308 Sharing Advances on Large Animal Models (http://www.salaam.genzentrum.lmu.de).

CONFLICT OF INTEREST

None of the authors has any conflict of interest to declare.

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