



## RESEARCH ARTICLE

### The expression of steroidogenic genes in ovine corpus luteum during early pregnancy

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### Erken gebelikte koyun korpus luteumunda steroidojenik genlerin ekspresyonu

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#### Öz

**Amaç:** Bu çalışmanın amacı erken gebelikte koyun korpus luteumunda steroidojenik genlerinin ekspresyonunu araştırmaktır.

**Gereç ve Yöntem:** Çalışmanın hayvan modelinde gebe koyunlar üç alt grup olacak şekilde dizayn edilmiştir; gebelik 12: G12, gebelik 16: G16 ve gebelik 22: G22. gün ve sıklık 16. gün (S16). Steroidojenik genlerin ekspresyon seviyesi (StAR, 3βHSD, P450scc) kantitatif polimeraz zincir reaksiyonu (PZR) ile değerlendirilmiştir ve StAR mRNA lokalizasyonu gebelik 16. günde sıklık 16. güne karşı in situ hibridizasyon yöntemi ile gösterilmiştir.

**Bulgular:** Gebelik günlerinde (G16 ve G22) StAR mRNA ekspresyonu G12 ile kıyaslandığında daha fazla olmasına ( $p<0.05$ ) karşın, G16 ve G22 günleri arasında fark bulunmamaktadır ( $p>0.05$ ). Benzer olarak, P450scc mRNA ekspresyon seviyesi gebelik günleri G22 ve G16'da G12'den fazla olarak bulunmuştur ( $p<0.05$ ). Ancak, P450scc mRNA ekspresyonunda G16 ve G22 günleri arasında fark bulunmamaktadır ( $p>0.05$ ). Benzer olarak, 3βHSD mRNA ekspresyon seviyesi gebelik günleri G22 ve G16'da G12'den daha fazladır ( $p<0.05$ ). Ancak, 3βHSD mRNA ekspresyonunda G16 ve G22 günleri arasında değişiklik tespit edilememiştir ( $p>0.05$ ). Bu çalışmadan, in situ hibridizasyonda, S16 günde StAR mRNA'sı luteal hücrelerde bulunamadı. Buna rağmen, luteal hücrelerde G16 günde StAR mRNA tespit edildi.

**Öneri:** Steroidojenik yolda bulunan genlerin ekspresyon seviyesinin koyunlarda erken gebelikte kritik bir rol oynadığı görülmüştür. Sonuç olarak, koyunların korpus luteumundaki steroidojenik yolağın, erken gebeliğin devamlılığı için gerekli progesteron sentezini transkripsiyonel olarak düzenleyebileceği önerilebilir.

**Anahtar kelimeler:** Korpus luteum, Erken gebelik, P450scc, StAR, 3βHSD

#### Abstract

**Aim:** The goal of this study was to investigate the expression of steroidogenic genes in ovine corpus luteum during early pregnancy

**Materials and Methods:** The animal model was designed as pregnancy; ewes were divided into three sub-groups, pregnancy 12th day: P12, pregnancy 16th day: P16, pregnancy 22th day: P22, and cyclic day 16 (C16). The expression of steroidogenic genes (steroidogenic acute regulatory protein; StAR, cytochrome P450 side-chain cleavage; P450scc, and 3β-hydroxysteroid dehydrogenase/delta5 delta4-isomerase; 3βHSD) was evaluated using qPCR, and mRNA localization of StAR was detected on P16 against C16 through in-situ hybridization.

**Results:** The expression of StAR mRNA was higher on day P22 and P16 compared to P12 ( $p<0.05$ ), while it was at a steady-state level on day P22 vs P16 ( $p>0.05$ ). mRNA expression of P450scc was greater on day P22 and P16 than day P12 ( $p<0.05$ ), however on day P22 vs P16, it was at a steady-state level ( $p>0.05$ ). Also, mRNA expression of 3βHSD had a similar trend; it was higher on day P22 and P16 compared to P12 ( $p<0.05$ ), while it was at a steady-state level on day P22 vs P16 ( $p>0.05$ ). In in-situ hybridization, we did not detect StAR mRNA on cyclic day 16 (C16) while abundantly expressed in luteal cells in P16.

**Conclusion:** The mRNA expression of steroidogenic genes may appear to play a critical role during early pregnancy in ewes. Accordingly, it can be suggested that the steroidogenic pathway in the corpus luteum of ewe may transcriptionally regulate progesterone synthesis required for maintenance of early pregnancy.

**Keywords:** Corpus luteum, Early pregnancy, P450scc, StAR, 3βHSD





## Introduction

Corpus luteum (CL) is regarded as a temporary endocrine gland, formed by the remnants of theca cells and granulosa layer of Graafian follicle after ovulation (Channing et al 1980, Sangha et al 2002). Steroidogenesis, the principal function of the CL, occurs in the luteal cells of which small cells more responsive to LH but large luteal cells primarily account for P4 synthesis and secretion of progesterone (P4) that is indispensable for the establishment and support of early pregnancy (Niswender et al 2000, Brooks et al 2014, Wiltbank et al 2018). Following ovulation, elevated concentrations of P4 augmented implantation and conceptus elongation in sheep (Kiyama et al 2016, Satterfield et al 2006) and dairy cows (Mann et al 2006); however, insufficient progesterone generation is the main reason of embryonic loss and infertility, as P4 action is necessary for both embryo growth and survival (Satterfield et al 2006).

During luteinization, a primary situation that occurs is that granulosa cells retain the capacity for extensive steroidogenesis by modulating machinery for the trafficking of cholesterol substrate out of the cytoplasm to mitochondria (Niswender 2002), which is the rate-limiting stage of hormone biosynthesis. Accordingly, *in vivo*, the essential molecule triggers for the stimulation of P4 is that the genes encoding the mechanism required for *de novo* synthesis of luteal steroids in the steroidogenic pathway, is expressed in the midcycle LH surge and early pregnancy (King and LaVoie et al 2012, Pokharel et al 2020).

Of these in the steroidogenic pathway, StAR have a significant role in regulating the transport of cholesterol to inner mitochondrial membrane of which generation of steroid is initiated (Stocco 2000, Christenson and Devoto 2003). The transcriptional regulation of most genes linked with synthesis of P4 initiates to upregulate and rises throughout the late luteal phase just as the CL has reached at fully maturation (Juengel et al 1995, Davis and LaVoie 2019). Also, P450scc and  $\beta$ HSD are among critical regulators associated with P4 biosynthesis. The P450scc enables the conversion of the newly transferred cholesterol to pregnenolone (King and LaVoie 2009). Consequently,  $\beta$ HSD facilitates in processing pregnenolone to P4 (Stouffer and Hennebold 2015, Plant et al 2015, Hu et al 2010).

Many species, including goats, pigs, and cows, need CL to generate progesterone for the vast majority of the pregnancy, while others such as primates and sheep only necessitate the CL for early pregnancy till the placenta is adequately formed to generate sufficient steroid (McCracken et al 1999, Magness 1998). Moreover, given that CL is transcriptionally regulated by control of gene expressions (Atli et al 2018) in response to maturation and early pregnancy (Hughes et al 2020, Kfir et al 2018), the main quest of this original study

is to test the expression of the steroidogenic genes in the CL during early pregnancy in ewes.

## Material and Methods

### *Animal diets and experimental design*

Animals were fed compliance with to National Research Council, 2007 (NRC 2007) according to nutritional necessities. The additional supplements and water were supplied *ad libitum*. For the current study, 2-5 aged multiparous Anatolian Merino ewes (n=12) were assigned into pregnant (on days of 12, 16, and 22; n = 4 per group) groups and corresponding cyclic day of 16 (C16) (Alak et al 2020, Eozenou et al 2020). In brief, following pre-synchronization, ewes were checked for their first natural estrus two times daily using a teaser ram and mated two times, every 12 hours, with rams having proven fertility. Then, the day of mating was recorded as day 0, and days 12, 16, and 22 were included as pregnancy groups (P12, P16, and P22). All ewes were slaughtered on days 12, 16, and 22 in pregnancy groups. The presence of embryonic trophoblast were ensured for 12, 16 and 22 days of pregnancy. CL tissue samples were obtained immediately and kept in cyro-tube at -80oC

### *RNA extraction and cDNA synthesis*

RNA of CL tissues were extracted as previously described (Hitit et al 2018). In brief, 40 mg of tissues in 1 mL TRIzol® were minced and 200  $\mu$ l chloroform poured to collect separation phase by centrifugation at 12000 RPM for 12 mins. Then, 400  $\mu$ l isopropanol were added, followed by collection of supernatant phase to precipitate RNA. Consequently, supernatant was discard and pellet washed twice through 70 % ethanol. RNA was eluted with 40  $\mu$ l of DEPC-dH<sub>2</sub>O and kept at -80°C until use. The concentration and quality of RNAs were checked using NanoDrop (Colibri Microvolume Spectrometer) evaluating the ratio of A260/A280 with 1.8–2.2 absorbance. A total of 1  $\mu$ g RNA was converted to cDNA using commercial kit (I-Script, BioRAD) by reverse transcriptase enzyme.

### *Gene expression*

Transrectal ovarian scans were conducted in all cows by ultrasonography (6.5-MHz linear-array transducer, KX5200-VET, Xuzhou Kaixin Electronic Instrument Co Ltd, Xuzhou, Jiangsu, China) to any CL present at PP 21 day and at the first day of the presynchronization protocol to determine cyclicity and the presence of CL was assessed as cyclic, non presence of CL was assessed noncyclic. During the ultrasound examination all cows were confirmed having no clinical abnormalities of the uterus or no abnormal vulva discharge (the presence of anechoic fluid in the uterine lumen, bioassay of potential endometritis). Pregnancy diagnosis was conducted

by transrectal ultrasonography on day 28-35 after TAI. Pregnancy was confirmed by anechoic uterine fluid and presence of an embryo with a heartbeat.

#### Assessment of *In situ* hybridization

The corpus luteum tissues for C16 (n = 4) and P16 (n = 4) were assessed for StAR, through *in situ* hybridization with techniques of free floating. All stages were performed according to previous work (Atli et al 2012). In short, low melting agarose was used to embed tissue sections and thickness of 22  $\mu$ m cut was performed using microtome (Leica VT1000 S, Germany). Primer sequence of StAR (Table 1) were employed to produce RNA probe templates of digoxigenin (DIG)-11-UTP-tagged. Following hybridization together with DIG tagged riboprobe, CL sections were used for incubation with anti-DIG. The bound probes were established and BM purple was utilized for an alkaline phosphatase chromogen to obtain DIG-tagged riboprobes. The images were taken with Nikon model of Eclipse E80i. (Nikon Instruments, NY, USA).

#### Gene ontology based network analysis

The gene interaction network was constructed based on Cytoscape with gene associations which had a confidence score  $\geq 0.4$ . The interactions were obtained from the types of evidence including experiments, databases, co-expression, and text mining limited to "Ovis aries". The KEGG pathway and GO enrichment were analyzed using Cytoscape software with the ClueGO V2.5.7 plug-in (Bindea et al 2009). The GO categories were allotted into cellular component, molecular function, and biological process. The P-value to 0.05 was set by two-sided hypergeometric tests, and Bonferroni step down adjustment was employed for multiple test correction. Kappa score threshold was set to 0.4.

#### Statistical analysis

The qPCR (Ct) data were calculated as relative expression as previously described (Hitit et al 2020). Using the  $2^{-\Delta\Delta Ct}$  method; the mean Ct values from the control sample were evaluated as the reference line, and the mean Ct values of pregnancy days were employed to calculate the relative expression from reference line (Livak and Schmittgen 2001). The normalized data were analyzed using IBM-SPSS (ver.21) with ANOVA and Tukey's post hoc test.  $p < 0.05$  was assessed as significant difference.

#### Results

The steady-state levels of luteal StAR mRNA during early pregnancy on day 12, 16, and 22 are shown in Figure 1. During early pregnancy, the level of StAR mRNA was found to be greater on P16 and P22 compared to P12 ( $p < 0.05$ ) whereas the level of StAR mRNA did not differ between P16 and P22 ( $p > 0.05$ ). We showed that mRNA of StAR was accurately localized to large luteal cells at P16, however StAR mRNA was not observed by *in situ* hybridization at C16 (Figure 2).

The steady-state levels of luteal P450scc mRNA during early pregnancy on day 12, 16, and 22 are demonstrated in Figure 3. During early pregnancy, the level of P450scc mRNA had higher expression levels on P16 and P22 compared to P12 ( $p < 0.05$ ) while the level of P450scc mRNA showed similar expression levels between P22 and P16 ( $p > 0.05$ ).

The steady-state levels of luteal  $3\beta$ HSD mRNA during early pregnancy on day 12, 16, and 22 are illustrated in Figure 4. During early pregnancy, the level of  $3\beta$ HSD mRNA was higher on P16 and P22 compared to P12 ( $p < 0.05$ ) while the level of  $3\beta$ HSD mRNA had similar expression levels between P22 and P16 ( $p > 0.05$ ).

Table 1. The gene primers used for qPCR and *in situ* hybridization

Gene ID	Primer sequence (5'-3')
StAR	CTCGCGACGTTTAAGCTGTG
( <i>For in situ</i> )	CGATGTTAATACGACTCACTATAGGGTCGTGAGTGATGACCGTGTC
StAR-F	GGACGAGGTGCTGAGTAAAG
StAR-R	CGCTCCACAAGCTCTTCATA
$3\beta$ HSD -F	AATCCGGGTGCTAGACAAAG
$3\beta$ HSD -R	CTCATCCAGAATGTCTCCTTCC
P450scc-F	GATGGCTCCAGAGGCAATAA
P450scc-R	CTGCTTGATGCGCTTGTC
YWHAZ-F	TGTAGGAGCCCGTAGGTCATCT
YWHAZ-R	TTCTCTCTGTATTCTCGAGCCATCT

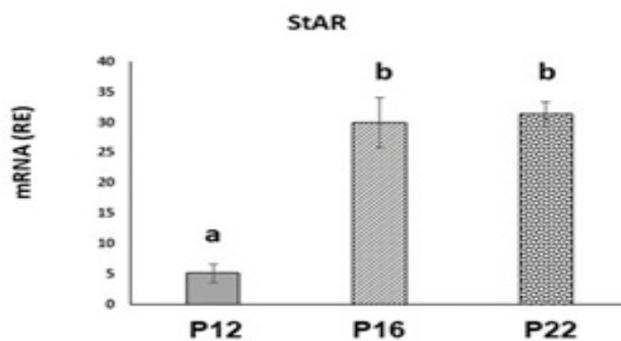


Figure 1. Expression of STAR mRNA on pregnancy days. P12: pregnancy 12th day, P16: pregnancy 16th day, P22: pregnancy 22th day. RE: relative expression. a, b: indicates significance ( $p < 0,05$ ).

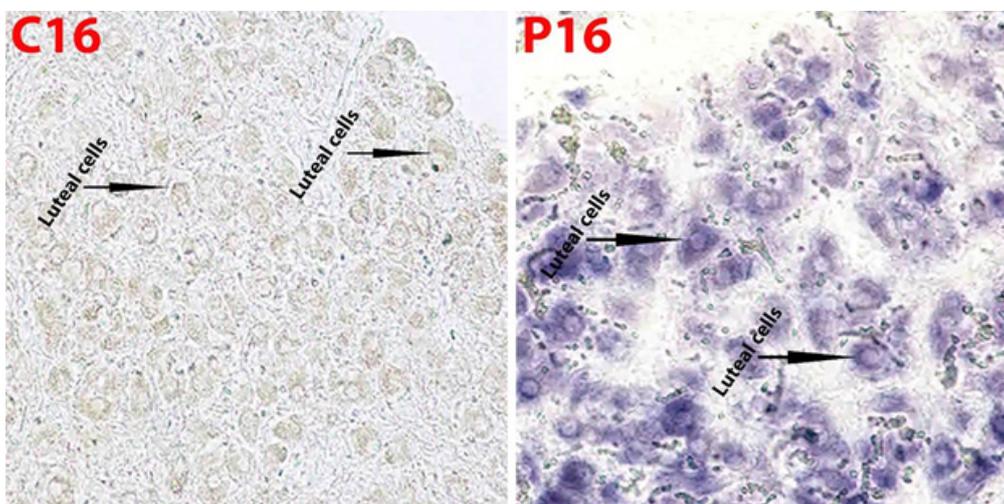


Figure 2. The representative images of Star mRNA in situ hybridization; A) ovine CL in C16 and B) ovine CL in P16. The black arrow indicates large cells.

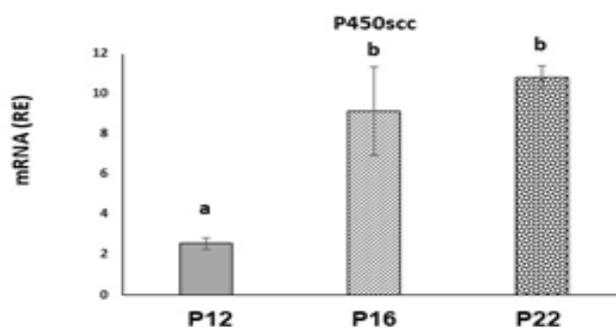


Figure 3. Expression of P450scc mRNA on pregnancy days. P12: pregnancy 12th day, P16: pregnancy 16th day, P22: pregnancy 22th day. RE: relative expression. a, b: indicates significance ( $p < 0,05$ ).



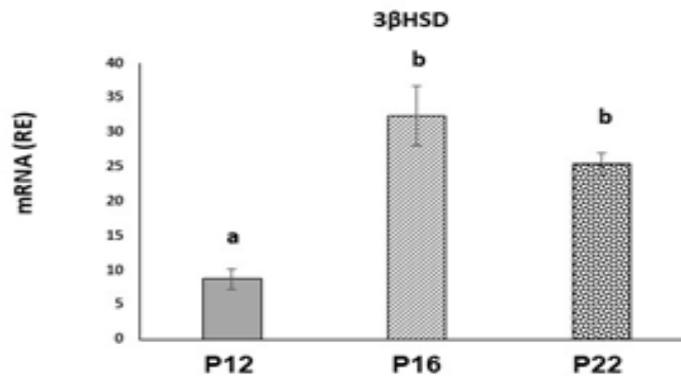


Figure 4. Expression of 3βHSD mRNA on pregnancy days. P12: pregnancy 12th day, P16: pregnancy 16th day, P22: pregnancy 22th day. RE: relative expression. a, b: indicates significance ( $p < 0,05$ ).

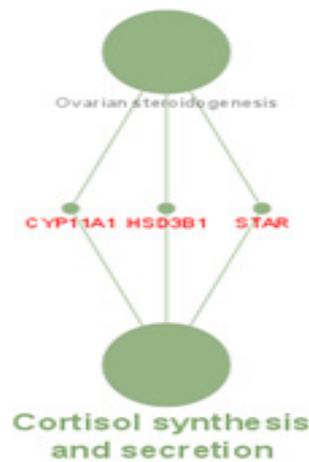


Figure 5. Gene ontology based network analysis. Functional interaction of network analysis of StAR, P450scc ve 3βHSD mRNAs in CL by ClueGO and associated genes were visualized using CluePedia. Pathway terms are displayed as nodes, the node color depth shows different proportions of proteins.

Results from the ClueGO on expressed genes (StAR, 3βHSD, P450scc) of steroidogenic genes were constructed and mRNA of StAR, 3βHSD, P450scc were consistently enriched in ovarian steroidogenesis pathway (KEGG:04927) identified in only cortisol synthesis and secretion network (Figure 5).

## Discussion

The current study clearly determined that the mRNA expression of some components of steroidogenic genes were investigated using qPCR in the ovine CL on day 12, 16, and 22 in pregnant ewes. The collection of CL in the ewes facilitated us to carefully detect the StAR, 3βHSD and P450scc expressions without any animal variation.

During luteal phase, in human, StAR expression is regulated and critically found to play role in CL formation while also involved in production of P4 throughout luteal cell survival (Devoto et al 2002, Kohen et al 2003, Sierralta et al 2005). Although StAR mRNA and protein expression was detected in theca cells in many mammalian species, it was not shown to be significantly expressed in granulosa cells (Bao and Garverick 1998, Bonnet et al 2008). In bovine, this consistent with that of early CL in granulosa cells which had weaker StAR mRNA expression while mid-cycle along with pregnancy showed greater expression (Hartung et al 1995). Moreover, StAR expression had positive correlation with P4 level in circulation (Yadav and Medhamurthy 2006). With respect to early pregnancy in pig, StAR expression found to be higher on day 14 of pregnancy than 12 in response to P4 increase (Przygodzka et al 2016).



In our study, concomitantly with above findings, we showed that StAR mRNA had greater expression in P16 and P22 than P12 in which steroidogenic activity is high in luteal cells. Accordingly, maintenance of P4 increment in response to presence of embryo may also contribute to increase of StAR mRNA on days of pregnancy P16 and P22.

The second stage in the P4 production is the catalysis of cholesterol to be converted into pregnenolone by P450scc enzyme, and consequent processing of pregnenolone into P4 by 3 $\beta$ -HSD. Along with StAR, P450scc expression was found to be gradually increased during days of 8-12th and reached at plateau levels in mid-luteal phase (Hartung et al 1995), showing similar patterns. The mRNA and protein expression of P450scc was at steady state level prior to LH surge in follicular theca cells and some granulosa cells and further upregulated during luteinization as well as maintained in functional CL; however, it decreased during luteal regression (King and LaVoie 2012). In very early study of ovine CL, mRNA of P450scc was shown not to differ between days of estrus cycle (3, 6, 9, 12, and 15th) vs days of 15 in pregnancy. However, similar to StAR expression, we found that P450scc mRNA was higher in P16 and P22 than in P12 in ovine CL. Considering embryo, our results were corroborated with that transgenic P450scc resulted in abnormal implantation due to misregulated P4 production (Chien et al 2013).

The expression levels of steroidogenic genes was related to production of progesterone (Mizutani et al 2015), and LH and exogen hCG are potentially induce transcription of StAR and P450scc as well as 3 $\beta$ HSD (King and LaVoie 2012). However, although P4 level were increased in hCG administered pregnant ewes, the expression of 3 $\beta$ HSD did not change (Colleson et al 2015). Also, 3 $\beta$ HSD was shown to be upregulated in pigs during early pregnancy (Przygodzka et al 2016). In the current study, we revealed that 3 $\beta$ HSD mRNA was found to be higher in P16 and P22 than in P12 in ovine CL while at steady state level between P16 and P22. This can explain that steroidogenic pathway is critically regulated in early pregnancy because luteal cell proliferation could enhance steroidogenic marker of 3 $\beta$ HSD upregulation (Yoshioka et al 2013).

## Conclusion

In recent years transcriptomic studies clearly revealed the importance of early pregnancy related genes for CL biology, thus critical for steroidogenic pathway in response to P4 control of pregnancy (Quan et al 2019, Hughes et al 2020). Considering the mRNA expression of components of steroidogenic genes and network analysis in ovarian steroidogenesis, they appeared to play critical role during early pregnancy in ewes, it can be suggested that steroidogenic pathway in corpus luteum of ewe may transcriptionally regulate progesterone synthesis required for maintenance of early pregnancy.

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## Conflict of Interest

The authors did not report any conflict of interest or financial support.

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### Ethical Approval

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