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# Interspecies pregnancy between *Ovis canadensis* mexicana and *Ovis aries* with reduced placental P450scc expression and intrauterine growth restriction

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## Abstract

The present study aimed to assess the steroidogenic process in the placenta of hybrids *Ovis canadensis mexicana* × *Ovis aries*, particularly, to determine the plasma lipid profile and the relative abundance of proteins related to the steroidogenesis, to elucidate in part the mechanism of progesterone deficiency in hybrids. Plasma lipoproteins and placental cholesterol were determined by dry chemical colorimetric assays. Steroidogenic acute regulatory protein (STARD1), StAR-related lipid transfer domain protein 3 (STARD3), cholesterol side-chain cleavage enzyme (P450ssc), and 3β-hydroxysteroid dehydrogenase (3β-HSD) were evaluated by Western blot. In addition, STARD3 was also investigated by immunohistochemical experiments in sheep placenta. Results indicated that cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and very low-density lipoprotein (VLDL) plasma levels were similar between hybrid and control pregnancy groups. Conversely, intracellular cholesterol was increased ($P < 0.05$) in the placenta tissue of hybrids. No significant differences in the relative abundance of both STARD3, STARD1, and 3β-HSD enzyme were observed in the placenta of hybrids compared to control animals, while the relative abundance of cytochrome P450scc was lower in hybrids than in control animals ($P = 0.0279$). The presence of STARD3 was observed in the cytoplasmic and perinuclear regions of mononucleate and binucleate cells of the trophoblast in the sheep placenta. The significant decrease in the abundance of P450scc in hybrids' placenta may explain the decrease in progesterone biosynthesis, which has been related to intrauterine growth restriction (IUGR) in these animals previously.

*Keywords:* Interspecies gestation; Pregnancy; Progesterone; Sheep; Steroidogenesis.

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## Study contribution

Progesterone is a steroid hormone that plays an important role in the regulation and maintenance of pregnancy, which is crucial for successful animal reproduction. Previous research conducted by our research team showed that hybrid sheep interspecies pregnancy was associated with intrauterine growth restriction. However, the mechanisms involved in progesterone deficiency in hybrid sheep gestations have not been elucidated. In this study, we demonstrated that progesterone biosynthesis and P450scc protein expression were both depleted in the placental tissue of hybrid sheep. These findings suggest that reduced P450scc protein expression in the hybrid placenta is responsible for the decrease in plasma progesterone levels, expanding our knowledge on this economically important topic.

## Introduction

Endocrine regulation of normal human fetal growth involves interactions between the mother, the placenta, and the fetus.[1, 2] Furthermore, one of the main causes of intrauterine growth restriction (IUGR) is a disrupted interaction between the three components, some of the risk factors for IUGR related to the mother include advanced maternal age, nutritional status during pregnancy, genetics, and health status before pregnancy[3, 4] Some of the fetal causes of IUGR are aneuploidy, congenital malformations, fetal infection, and genetics, among others.[4]

Mother and fetus are physiologically interrelated by the placenta, the essential organ that regulates the exchange of nutrients and gases and the removal of cell detritus in this binomial. Likewise, the placenta is an endocrine organ that produces essential hormones for fetal growth and development.[5, 6] In this sense, any change in hormone synthesis or the metabolism of placental nutrients influences the rate of fetal growth,[5, 7] like the IUGR.[2, 8]

The placenta is the primary source of steroid hormones biosynthesis during pregnancy, including estrogens and progestogens.[9, 10] Progesterone is the main progestogen synthesized, playing a major role in the regulation and maintenance of gestation,[11–14] and exerting other functions, such as promoting uterine growth and suppressing myometrial contractility.[12] In addition, high levels of progesterone induce the mobilization of fatty acids and glucose from body storage to maternal circulation, which is used later by the fetus via the placenta.[15]

Studies with mice and sheep have documented the association between IUGR and placental insufficiency with reduced progesterone levels in hybrid pregnancy contexts.[16, 17] Studies identified at least 6 hormones dysregulated in the case of IUGR, particularly, progesterone.[18] Therefore, progesterone has an essential role in maintaining gestation and the health of the fetus.[19, 20] The placenta requires cholesterol for progesterone biosynthesis, and during pregnancy, its primary source comes from maternal serum cholesterol, LDL, and HDL.[21, 22] In ruminants, plasma cholesterol levels are influenced by different factors, such as diet, age, sex, breed, season, pregnancy, lactation, diseases of the liver, biliary tract and by the number of deliveries.[23, 24]

In sheep, the synthesis of ovarian progesterone by the corpus luteum is essential for the maintenance of early pregnancy, but as pregnancy progresses, the placenta becomes the main source of progesterone.[8, 25] Steroid hormones are

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synthesized from cholesterol, which is transported to the mitochondria of specialized cells of the adrenal glands, gonads, and placenta by sterol transport proteins called START proteins. The first of these proteins is the steroidogenic acute regulatory protein (STARD1), which is found in adrenal glands and gonads, and the second is the StAR-related lipid transfer domain protein 3 (STARD3), which is found in the human placenta.[26, 27] Because the expression of STARD3 in sheep placenta has not been previously demonstrated, in this study we investigated its expression and tissue localization by immunohistochemistry.

In the placenta, as in other steroid-synthesizing tissues, cholesterol is transformed to pregnenolone within the mitochondrial membrane by cholesterol side-chain cleavage enzyme (P450scc). This conversion is a reaction of the P450scc enzyme and is the first enzymatic step in steroid synthesis, then, pregnenolone is converted to progesterone by the 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD) enzyme.[28–30]

Previous studies have demonstrated a reduction in the plasma concentration of progesterone during the last two-thirds of gestation in a hybrid of *Ovis canadensis mexicana* × *Ovis aries*, importantly, reduced progesterone was associated with decreased placental and cotyledon weights and IUGR of lambs.[17, 31, 32] However, the mechanisms involved in progesterone deficiency in these gestations have not been elucidated. This study evaluated the plasma lipid profile and the relative abundance of proteins associated with the placental steroidogenic process at term to elucidate the alterations responsible for reduced progesterone biosynthesis pathways in hybrid sheep.

## Materials and methods

### *Ethical statement*

The care and handling of animals was approved by the Internal Subcommittee for the Care and Use of Animals for Experimentation (SICUAE), of the Faculty of Veterinary Medicine and Zootechnics (SICUAE.DC-2013/1-7), and performed according to the Official Mexican Standards (NOM-O62-ZOO-1999).

### *Animals and experimental design*

Ten Suffolk sheep aged 2–3 years were used for this study. They were divided into two groups, each group consisting of four pregnant ewes with a single fetus. 1) hybrid pregnancy *Ovis canadensis mexicana* × *Ovis aries*, (n = 4) and 2) control group *Ovis aries* × *Ovis aries*, (n = 4). In addition, two embryo donor ewes, synchronized to obtain animals with superovulation, were used as a source for obtaining embryos that were transferred individually to ensure the pregnancy of a single lamb per ewe in each group.[31]

Throughout the experiment, the feed was offered *ad libitum* in a group feeder, allocated for pregnant ewes: during the first two-thirds of gestation, ewes received 1.75 kg of dry matter, 3.35 MCal of metabolizable energy and 96 g of metabolizable protein. During the last third of gestation, ewes received 2.15 kg of dry matter, 4.10 MCal of metabolizable energy, and 124 g of metabolizable protein. These

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needs were covered with oat hay, alfalfa, and concentrate feed. Water was also supplied *ad libitum* throughout the experiment.[17]

## Hybrid pregnancy group

After synchronization and superovulation induction, using vaginal sponges with 20 mg of fluorogestone and 200 IU of follicle-stimulating hormone (FSH), respectively,[33] the donors were inseminated (by laparoscopy) with two samples of frozen semen from Bighorn sheep (*Ovis canadensis mexicana*), as previously reported.[31] Six days after insemination, hybrid embryos were collected and individually transferred by laparoscopy into recipient sheep *Ovis aries*.[33]

## Control group

Animals in the control group were allowed to mate with a fertile ram *Ovis aries*, as described previously.[32] At day 50 after insemination, pregnancy was confirmed by ultrasound images.

## Biological samples

Placental samples were collected immediately after normal delivery.[26, 34] Next, they were placed and washed in phosphate-buffered saline (PBS) at 4 °C (137 mM NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO4$, $KH_2PO4$, pH = 7.4), and tissue debris was removed. To avoid degradation, random samples of placental cotyledon tissue (12 small pieces, approximately 1–2 cm in diameter) were immediately frozen at -70 °C until the analyses were performed.[34] Tissue samples were processed for Western blot analysis and determination of cholesterol in placental tissue.

Briefly, tissue samples were placed in a lysis buffer with protease inhibitors (0.1 % sodium dodecyl sulfate, 0.5 % sodium deoxycholate, 1 % NP-40, 150 mM sodium chloride, 50 mM Tris-HCl, pH = 8), and stored at -70 °C until its use.[35] Other groups of tissue samples were fixed in PBS paraformaldehyde 4 % solution at 4 °C overnight and subsequently embedded in paraffin for immunohistochemical studies. Blood samples were collected by venipuncture into tubes containing heparin and centrifuged at 1 500 g for 10 min at 4 °C. The plasma was then carefully transferred to 1.5 mL tubes and stored at -20 °C until analysis.[32]

## Plasma lipid profile assay

The plasma lipid profile was determined at 12, 16, and 20 weeks of gestation. Free cholesterol, triglycerides, and high-density lipoprotein (HDL) values were determined by enzymatic colorimetric methods, according to the manufacturer's recommendations. The analyses were performed in a semi-automatic chemistry analyzer ORTHOS CLINICAL DIAGNOSTICS (Vitros 5600). Very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) values were calculated using the Freidewald formula [VLDL = division of triglycerides by 5, while LDL = total cholesterol - HDL - VLDL].[36]

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All samples analyzed were processed in duplicate (technical replicates) and reported as a mean value. All measurements were performed with the same conditions, and processed in the same batch to avoid inter-assay variations. The coefficient of internal variation was considered concerning the mean value of the quality controls with less than one standard deviation.

## *Western blot*

Biological replicates of placental tissue from each animal, obtained immediately after delivery, were analyzed. The dissected tissue from the placenta was homogenized in lysis buffer with the homogenizer (Kinematica Polytron PT 2100) at 3 000 rpm for 3 min (20 s on/off intervals). The sample was then sonicated at 65 % amplitude for 3 min (20/60 s on/off intervals) at 4 °C and centrifuged at 5 000 g for 15 min at 4 °C. The supernatant was recovered, and the total protein was determined by the Lowry method.[37] From each condition, 80 µg of protein were suspended in loading buffer (5 % β-mercaptoethanol, 0.1 % glycerol, and 0.01 % bromophenol blue), and loaded into 10 % SDS-polyacrylamide gel.

Protein separation was performed under denaturing conditions and subsequently transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked with 5 % w/v nonfat dry milk and 0.1 % v/v Tween-20 at room temperature for 60 min.[37] Blocked membranes were incubated at 1:1 000 with primary antibodies against StAR-related lipid transfer domain protein 3 (STARD3) (sc-292868), steroidogenic acute regulatory protein (STARD1) (sc-25806), 3β-hydroxysteroid dehydrogenase (3β-HSD) (sc-28206), cholesterol side-chain cleavage enzyme (P450scc) (sc-292456), or at 1:10 000 (β-actin) (sc-47778) at 4 °C overnight.

Membranes were then incubated with the corresponding secondary antibodies conjugated to horseradish peroxidase (HRP): anti-mouse at 1:10 000 (sc-2302) or anti-rabbit at 1:20 000 (sc-2301). The signal was detected by chemiluminescence using the ECL-Plus detection system (Millipore Corporation, MA, USA) with a blot scanner. Densitometry analysis was performed with the Image Studio Lite version 3.1 (RRID: SCR_013715), normalized against β-actin.

## *Immunohistochemistry*

Three independent placental tissue sections (5 µm) from each animal were analyzed, and the samples were uniformly oriented. Paraffin-embedded sections were dewaxed and rehydrated in decreasing concentrations of ethanol. Antigen retrieval was achieved by boiling samples in 0.01 mol/L sodium citrate (pH = 6) for 20-40 min. Slides were incubated with 2 % bovine serum albumin (BSA) solution for 2 h at room temperature and extensively washed.[38] Slides were then incubated at 4 °C in a humid chamber overnight with primary antibody anti-STARD3 (sc-292868) diluted 1:100 in PBS containing 1 % BSA.

The primary antibody was omitted in the negative controls. Quenching of endogenous peroxidase was achieved by incubation with 0.3 % hydrogen peroxide in methanol for 30 min at room temperature. Slides were incubated with HRP-conjugated anti-rabbit antibody (sc-2301) diluted 1:300 in PBS containing 1 % BSA for 1 h at room temperature. The reaction was detected using the Diaminobenzidine

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Peroxidase HRP substrate Kit (SK-4100) from Vector Laboratories according to the manufacturer's instructions. Finally, samples were evaluated with a Nikon Eclipse E600 microscope (Nikon Instruments Inc., Melville, NY, USA).

## *Determination of cholesterol in placental tissue*

Cholesterol content was determined from placental tissue samples collected immediately after delivery by homogenization of placental tissue in lysis buffer as previously described and was determined spectrophotometrically at 25 °C using the Cholesterol LQ kit (Spinreact, México) according to the manufacturer's instructions. Three replicates were performed for each ewe. The analytical sensitivity was 1 mg/dL = 0.0019 absorbance. All measurements were performed with the same conditions, processed in the same batch to avoid variations, in an Agilent 8453 UV–visible spectrophotometer.[39]

Plasma progesterone levels in the second and third of gestation and weight of placenta and lambs at birth in the hybrid gestation and control groups. The plasma levels of progesterone, as well as the morphological characteristics of the lambs at birth, were variables evaluated and their results were published in previous studies, by our study group.[32] During the last two-thirds of gestation, plasma progesterone levels were measured weekly in ewes of the hybrid gestation group and the control group. Differences between groups were observed at weeks 8, 14, and 16 to 21 (Table 1).

**Table 1.** Plasma progesterone levels in the last two-thirds of pregnancy in control and hybrid ewes

| Week of gestation | Control group (n = 6) Mean ffl SEM (ng/mL) | Hybrid group (n = 4) Mean ffl SEM (ng/mL) | P-value |
|---|---|---|---|
| Week 8 | 9.4 ± 1.5 | 4.6 ± 0.8 | 0.0384 |
| Week 9 | 8.3 ± 1 | 6.2 ± 1 | 0.1989 |
| Week 10 | 7.4 ± 1.1 | 6.5 ± 0.7 | 0.5782 |
| Week 11 | 10 ± 1.9 | 10 ± 3.7 | 0.9906 |
| Week 12 | 9.5 ± 1.9 | 7.2 ± 0.8 | 0.3873 |
| Week 13 | 9.3 ± 1.2 | 6.0 ± 0.7 | 0.0782 |
| Week 14 | 10.3 ± 1.1 | 6.7 ± 0.5 | 0.0326 |
| Week 15 | 14.4 ± 2.3 | 8 ± 1.1 | 0.0696 |
| Week 16 | 17.2 ± 2.1 | 8.2 ± 1 | 0.0119 |
| Week 17 | 22.2 ± 2.5 | 8.9 ± 1.4 | 0.0042 |
| Week 18 | 22.6 ± 2.2 | 8.3 ± 0.7 | 0.0031 |
| Week 19 | 24.9 ± 1.1 | 8.5 ± 1.1 | 0.0001 |
| Week 20 | 22.8 ± 1.1 | 8.2 ± 0.4 | 0.0001 |
| Week 21 | 22.8 ± 6 | 7.7 ± 1.5 | 0.0314 |

Pregnancy in control: [(mean ± standard error of the mean (SEM)]. Modified from Chávez-García et al.[32] Permission (5677771260233).

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Birth weights of offspring from hybrid gestations (n = 4) and offspring from control group gestations (n = 6) and their respective placentas. A total of 12 cotyledons were weighed from each placenta of the hybrid group and the control group (Table 2).

**Table 2.** Characteristics of the offspring after birth in control and hybrid ewes

| Weight | Control group (n = 6) Mean ffl SEM | Hybrid group (n = 4) Mean ffl SEM | P-value |
|---|---|---|---|
| Weight of lambs (kg) | 5.5 ± 0.3 | 2.4 ± 0.2 | 0.0001 |
| Weight of cotyledons (g) | 35 ± 1.8 | 17.9 ± 2.1 | 0.0003 |
| Weight of placentas (kg) | 0.6 ± 0.1 | 0.3 ± 0.1 | 0.0036 |

Offspring after birth: [(mean ± standard error of the mean (SEM)]. Modified from Chávez-García et al.[32] Permission (5677771260233).

### *Statistical analysis*

Statistical analysis was performed using the Graph Pad Prism software (RRID: SCR_002798). The unpaired t-test was used to compare group means and determine if there was a significant difference in lipoproteins, cholesterol, tissue cholesterol, STARD1 and STARD3 proteins as well as P450 and 3β-HSD enzymes. The significance level was set at $P < 0.05$.

## Results

### *Cholesterol and lipoprotein levels*

We observed increased plasma HDL levels in ewes from the hybrid group compared with the control group ($P = 0.0243$), only at 16 weeks of gestation (Figure 1). Plasma levels of cholesterol, LDL, and VLDL showed no differences between the control and hybrid groups (Figure 1).

### *Cholesterol content in placental tissue*

Cholesterol content was more than 40 % higher ($P < 0.0001$) in the placental tissue of the hybrids compared with the control group (Figure 2).

### *Expression of enzymes associated with steroidogenesis in placental tissue*

Figure 3 shows densitometry and representative Western blots of specific steroidogenic enzymes (STARD1, STARD3, and 3β-HSD) from placental tissue collected after delivery from hybrid and control groups. P450scc in the hybrid group compared with the control group ($P = 0.0279$).

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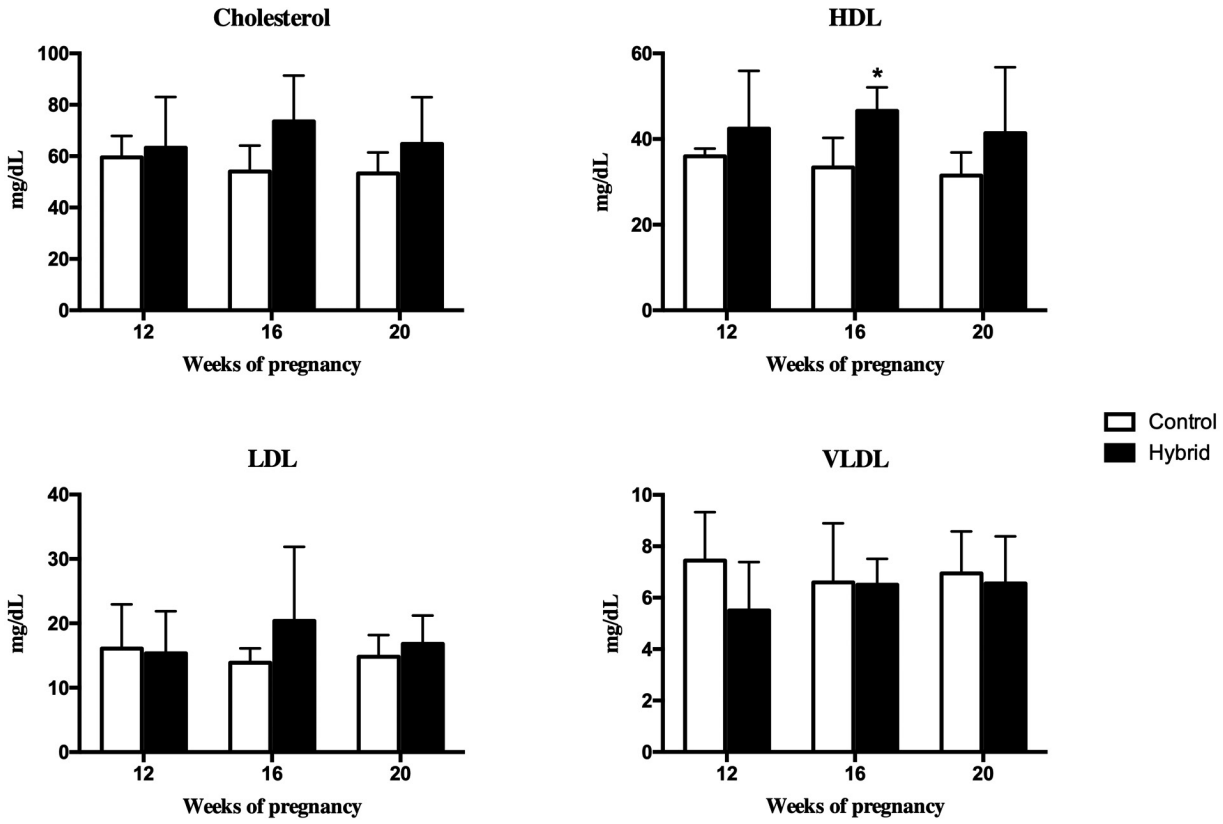


Figure 1. Mean ± standard error of mean plasma cholesterol and lipoprotein levels (mg/dL), in the hybrid (n = 4), and control (n = 4) groups at 12, 16, and 20 weeks of gestation. *Denotes a statistically significant difference ($P < 0.05$).

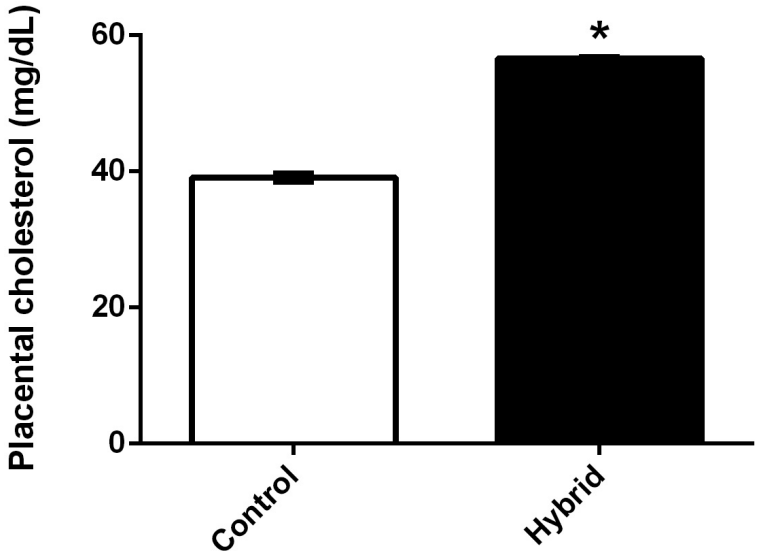


Figure 2. Mean ± standard error of the mean for cholesterol levels (mg/dL) in placental tissue (cotyledons) after delivery of the hybrid group (n = 4), and the control group (n = 4). *Denotes a statistically significant difference ($P < 0.05$).

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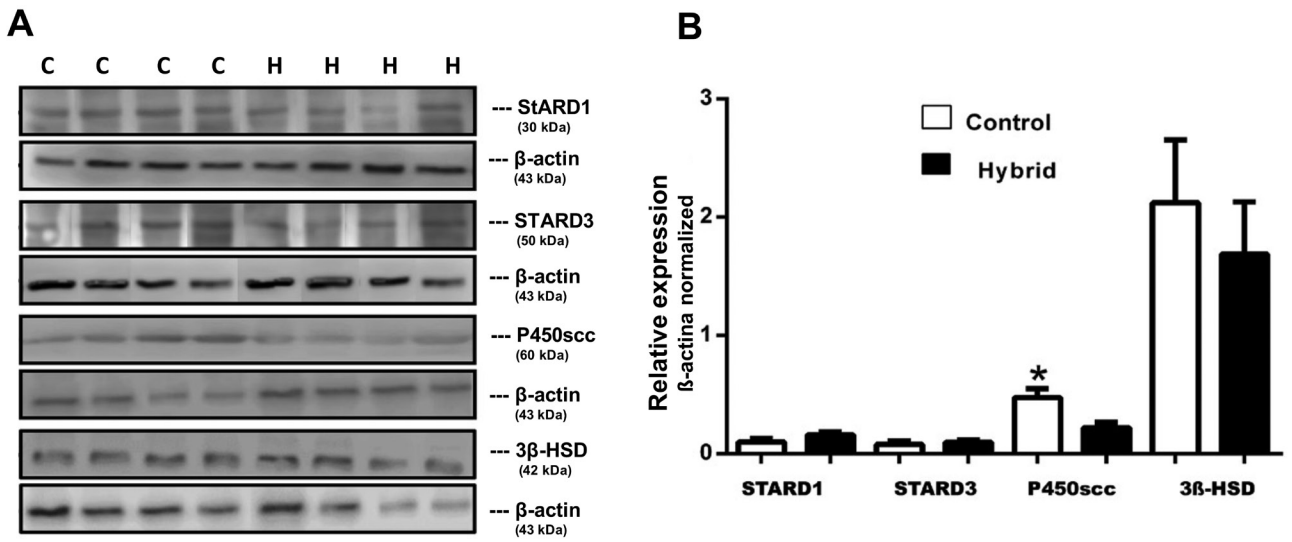


**Figure 3.** (A) Densitometry analysis of Western blot specific protein bands of steroidogenic enzymes in placenta of hybrid group (H, n = 4), and control group (C, n = 4). (B) Mean ± standard error of the mean for protein expression. Steroidogenic acute regulatory protein (STARD1), StAR-related lipid transfer domain protein 3 (STARD3), cholesterol side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), kilodaltons (kDa). *Denotes a statistically significant difference ($P < 0.05$).

### *Expression and localization of the enzyme STARD3*

Since STARD3 expression in ovine placenta has not been previously demonstrated, we examined its expression and tissue localization by immunohistochemistry only in the control group as a confirmatory study (Figure 4).

## Discussion

In this study, we found expression of both STARD1 and STARD3 in placental tissue, but no differences were found between pregnancies in the control and hybrid groups. On the contrary, a higher intracellular cholesterol content was found in the placental tissue of the hybrid pregnancies. However, there were no differences in maternal plasma cholesterol levels between the groups. Interestingly, at 16 weeks of pregnancy, a significant increase in plasma HDL levels was observed in ewes of the hybrid group. This difference may be due to the changes in liver metabolism during this time of gestation.[23] However, the observed increase returned to normal levels in the subsequent weeks.

The increased cholesterol found in placental tissue in the present study suggests that it was a mechanism for attempting to restore normal progesterone biosynthesis in response to the reduced levels of this hormone found in ewes with interspecies pregnancy.[17, 32] These data suggest that changes in progesterone biosynthesis previously observed,[32] in pregnant ewes with hybrids, were not due to a deficiency in the cholesterol supply. The mitochondrial cholesterol supplier proteins are STARD1 and STARD3, both of which are observed in steroidogenic tissues in humans.[40, 41] In addition, it has been demonstrated that the expression of STARD1 enzyme in the sheep placenta and its expression had a significant increase

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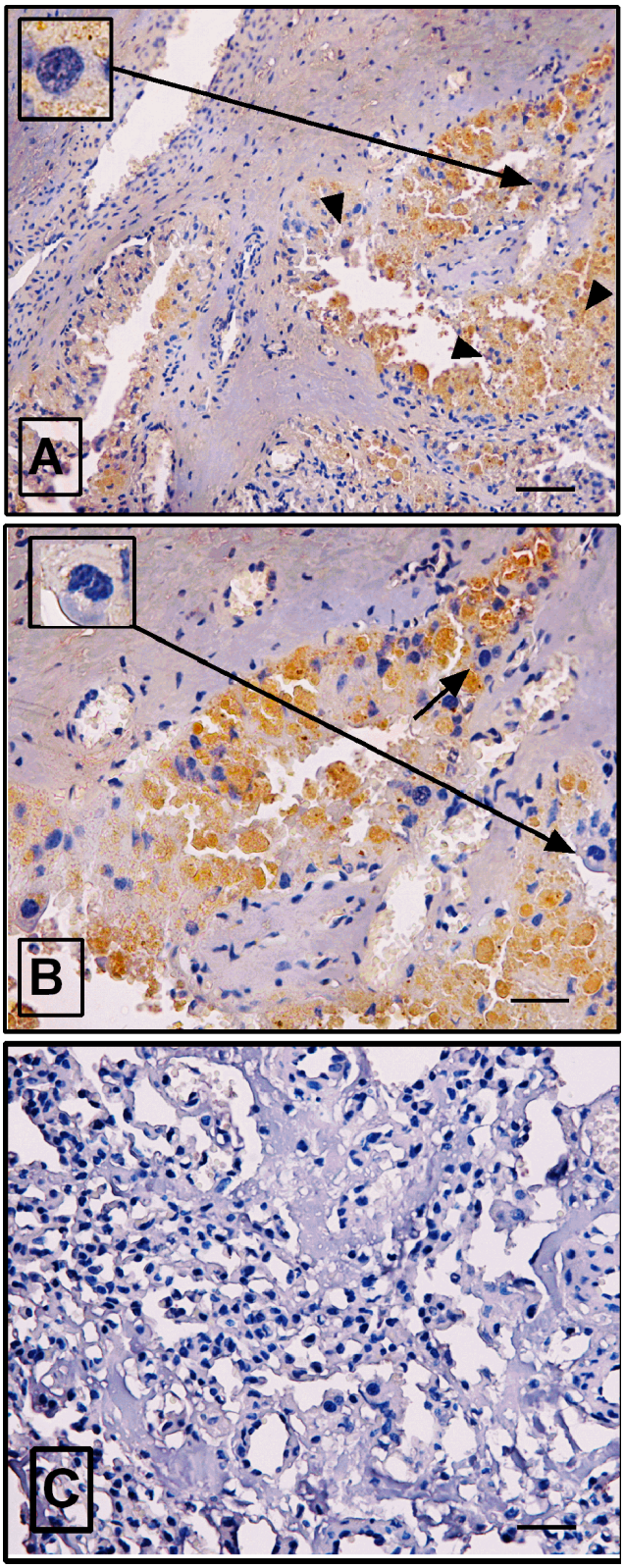


Figure 4. Immunohistochemical analysis of STARD3 in placental tissue from control group. (A) arrowheads show positively stained mononuclear cells; upper box shows magnification. (B) Arrows show positively stained binuclear cells, upper box shows magnification. (C) Control immunohistochemical staining (without primary antibody). Scale bar 100 µm.

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during fetal development, which correlates with the increased of steroid production by the placenta at the end of gestation.[25] In other studies in ewes, RNA transcripts for the STARD1 enzyme was detected in placental samples at mid and late gestation (day 81 and day 130).[8]

In addition, we identified the expression of STARD3 protein in placental tissue and observed that it is present in both mononucleate and binucleate trophoblast cells. In previous studies, it has been demonstrated that STARD3 induces cholesterol accumulation in endosomes at the expense of the plasma membrane and that it participates in the distribution of intracellular cholesterol through its transport from the endoplasmic reticulum to endosomes.[42, 43] In the present study, accumulation of STARD3 around the nucleus were observed in mononucleate and binucleate cells, suggesting that it localizes in late endosomes of perinuclear clouds, which is in line with previous studies regarding STARD3 expression and accumulation in late endosomes in humans.[44, 45] To our knowledge, this is the first comprehensive study of the immunohistochemical localization of STARD3 in ovine placenta. Further studies are needed to determine its contribution to steroidogenesis in the ovine placenta in addition to STARD1.

The rate-determining step of human placental progesterone synthesis is the conversion of cholesterol to pregnenolone by cytochrome P450scc.[40, 41] In this study, we observed that P450scc protein levels were significantly decreased in placental tissue from hybrid gestation ewes compared with controls, suggesting that the activity of this important limiting step of steroid biosynthesis may be impaired leading to a significant decrease in progesterone biosynthesis observed in previous studies,[17, 32] but further experimental evidence is required to support this relationship. However, in another study model, but also in pregnant ewes, decreased expression of the P450scc enzyme was observed in placental tissue, which was associated with decreased progesterone biosynthesis and IUGR, similar to the hybrid pregnancies in our study.[8]

It is well known that pregnenolone produced by the activity of P450scc is converted to progesterone by 3β-HSD type 1 enzyme in the placenta.[41, 46] However, in the present study, we did not find significant differences in the 3β-HSD protein content between the hybrid and control groups, suggesting that the previously observed reduction in progesterone is not associated with a decrease in the expression of this enzyme. In addition, previous studies have shown that sheep placenta produce large amounts of this enzyme.[8, 10] The results presented suggest that progesterone serum levels reduction in maternal circulation is associated with a decrease in placental P450ssc protein content, and this could be the reason of the IUGR observed in hybrid animals and other models,[8, 10, 17, 32] however, further studies should be done to determine the molecular mechanism of P450ssc protein reduction and its relation with IUGR.

## Conclusions

The results indicated that during the pregnancies of the hybrid group and the control group, plasma cholesterol and lipoprotein levels were similar; however, cholesterol in the placental tissue of the hybrid pregnancy group increased significantly,

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suggesting an effort to restore normal progesterone levels. Furthermore, in this study, both STARD1 and STARD3 proteins were expressed in the placenta of ewes, and the levels did not change between the hybrids and the control. However, a significant decrease in P450scc protein expression was observed in placental tissue from hybrid pregnancies, suggesting that this is responsible for the decrease in plasma progesterone. Further studies are needed to elucidate the molecular mechanisms involved in the regulation of P450scc protein in hybrid sheep placenta.

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## Data availability

The original datasets used in this research and if applicable, supporting information files, are deposited and available for download at the SciELO Dataverse repository.

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## Conflicts of interest

The authors have no conflict of interest to declare in regard to this publication.

## Author contributions

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Writing-review and editing: A. Chávez-García, EA. Cabrera-Reyes, G. Molina-Salinas, S. Olvera-Sánchez, F. Martínez, M. Cerbón.

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