**Phenotypic and molecular characterization of intrauterine fetal growth restriction in interspecies sheep pregnancy**

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**ABSTRACT:** Interspecies pregnancies between closely related species are usually performed in livestock to obtain improved and enriched offspring. Indeed, different hybrids have been obtained for research purposes since many years ago, and the maternal–fetal interactions have been studied as a possible strategy for species preservation. The aim of this study was to characterize by physiological and molecular approaches the interspecies pregnancy between bighorn sheep (*Ovis canadensis mexicana*) and domestic sheep (*Ovis aries*). Hybrids were obtained by artificial insemination; the blood pressure and protein urine levels were measured during the last two-thirds of gestation. After parturition, offspring and placentas were weighed and measured and cotyledons were counted and weighed and their surface area determined. Plasma samples were obtained between wk 8 and 21 of gestation to assess progesterone (P4), vascular endothelial growth factor (VEGF), and placental growth factor (PlGF) levels and cell-free RNA was isolated during the same period to assess hypoxia-inducible factor-1 α (*HIF-1α*) gene expression. Hybrid and normal pregnancies were analyzed using physiological and molecular parameters during the last two-thirds of gestation (wk 8–21). The results show that during the measurement period, ewes with a hybrid pregnancy presented normal blood pressure and no alteration in urinary protein content. However, compared with sheep with a normal pregnancy, those with a hybrid pregnancy had a decrease in fetal and placental growth as well as in the cotyledonary surface area. Furthermore, in the hybrid group, there was placental insufficiency, characterized by a decrease in P4 production, as well as indications of endothelial dysfunction, characterized an increase in plasma levels of VEGF and PlGF as well as in plasma gene expression of *HIF-1α*. Overall, the results indicate that hybrids of *O. canadensis mexicana* and *Ovis aries* presented intrauterine growth restriction, essentially due to altered endothelial function and chronic placental insufficiency. Further studies are necessary to overcome this primary placental dysfunction and thus obtain improved offspring for future molecular and genomic evaluations.

**Key words:** endothelial dysfunction, fetal growth restriction, hybrid, intrauterine growth restriction, placental insufficiency, pregnancy


**INTRODUCTION**

Interspecies pregnancies have been achieved with the expectation of obtaining new genetically improved offspring (Verkaar et al., 2003; Sumar, 2013). Although this strategy has been used mainly to obtain better livestock species, it is also used to preserve endangered species (Lanza et al., 2000). Previous studies in this field have described different analyses including physiopathology, pharmacology, and genetics of both pregnancy physiology and maternal–fetal interactions in hybrids.
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(Musa et al., 2012; Widayati, 2012). *Ovis canadensis mexicana* (bighorn sheep) is considered one of the most important endangered mammals in Mexico (NOM-059-ECOL-1994 and NOM-059-SEMARNAT-2010, Environment and Natural Resource Ministry); therefore, it represents a perfect candidate for the study of hybrids as a strategy to ensure its conservation.

To characterize interspecies pregnancies, it is necessary to analyze fetal development, womb angiogenesis, and other pregnancy-related markers in the ewe’s serum.

A healthy pregnancy and adequate fetal growth require normal placental development, which, in turn, depends on the proper establishment of placental angiogenesis (Ahmed et al., 2000; Myatt, 2006; Zhang et al., 2013). On the other hand, progesterone (P4) is unequivocally necessary for maternal support of the conceptus survival and development (Spencer et al., 2004; Song et al., 2008). In addition, angiogenesis requires an adequate oxygenation in fetoplacental vessels. Hypoxia-inducible factor (HIF-1) is a master regulator of the cellular response to low oxygen tension and is central in the maintenance of oxygen homeostasis (Ahmed and Perkins, 2000; Gourvas et al., 2012a,b).

The aim of the present study was to characterize the physiological and molecular changes that take place during the last two-thirds of the interspecies pregnancy resulting from the crossing of bighorn sheep (*O. canadensis mexicana*) and domestic sheep (*O. aries*).

### MATERIAL AND METHODS

#### Animals

The handling and care of animals was approved by the Internal Subcommittee for the Care and Use of Animals for Experimentation and was in accordance with the official Mexican standards (NOM-O62-ZOO-1999). The study was conducted from August 2012 to February 2013 at the Center for Teaching, Research and Outreach in Sheep Production, located in the town of Tres Marías, in the state of Morelos, Mexico.

This center belongs to the Faculty of Veterinary Medicine and Animal Husbandry of the National Autonomous University of Mexico.

A total of 14 crossbred Suffolk ewes, ranging from 2 to 5 yr of age, were used in the study. Animals were fed daily with oat hay, silage corn, and commercial feed. The ewes were randomly divided into the hybrid and control groups. The hybrid group was subdivided into the donor and recipient groups. After synchronization and superovulation induction (controlled internal drug release device and 200 UI of FSH, respectively; Tinajero et al., 2005; Mejia et al., 1998), the donors were inseminated with 2 samples of frozen bighorn sheep (*O. canadensis mexicana*) semen by laparoscopy, as previously reported (Ramírez-Molina et al., 2005). At 6 d after insemination, hybrid embryos were collected as previously described (Mejia et al., 1998).

To avoid multiple gestations, the embryos were individually transferred to synchronize recipient ewes by laparoscopy (Mejia et al., 1998; Ramírez-Molina et al., 2005). On the other hand, the sheep control group was allowed to mate with a fertile ram (*O. aries*), as the semen samples for artificial insemination cannot be easily collected from wild males.

At Day 50 after insemination or after mating, pregnancy diagnosis was conducted by ultrasound imaging. Multiple pregnancies were excluded from the study. There were 6 pregnant ewes in the control group and 4 in the hybrid group.

#### Parameters and Biological Samples

All assays were performed weekly after the 50th day of pregnancy. Blood samples were collected by venipuncture in tubes containing EDTA and heparin and were centrifuged at 1,500 × g for 10 min at 8°C. Then, plasma was carefully transferred into 1.5-mL tubes and stored at −20°C until assayed to measure P4, vascular endothelial growth factor (VEGF), placental growth factor (PIGF), and cellular free HIF-1α mRNA levels. The VEGF and PIGF levels were obtained by ELISA, using a commercial kit and following the manufacturer’s recommendations (900k10; Peprotech México, SA de CV, Mexico), as previously validated and reported (Gómez et al., personal communication). The sensitivity of the method for measuring VEGF was 3.58 pg/mL and the intra-assay variation coefficient was 8.26%. The detection limit for PIGF was 2.15 pg/mL and the intra-assay variation coefficient was 6.85% (Gómez et al., personal communication). A solid-phase RIA (Pulido et al., 1991) was performed for P4 measurement. The sensitivity of the method was 0.1 ng/mL and the intra-assay variation coefficient was 3.09%.

Blood pressure was obtained weekly from the median artery (left member) by using an automatic digital wrist blood pressure device (Microlife BP 3BUI EMT-3; Mucha, 2007). The mean arterial pressure (MAP) was calculated with the following formula: \( \text{MAP} = (\text{SP} – \text{DP})/3 + \text{DP} \), in which SP is the systolic blood pressure and DP is the diastolic blood pressure (Acoltzin-Vidal et al., 2010). Importantly, MAP assessment was performed with a minimum of handling to reduce the stress reaction alert in females.

Proteinuria was determined using specific and sensitive albumin test strips, according to the manufactur-
er’s instructions (Mission Laboratories, Inc., ACCON; Campuzano and Arbeláez, 2007). The weight of offspring was determined at birth, at which time the placenta was recovered and weighed. Twelve cotyledons were then randomly taken to determine their weight and surface area.

Total RNA was isolated with TRIzol reagent (Life Technologies,) and RT-PCR was performed using Moloney Murine Leukemia Virus Reverse Transcriptase (Life Technologies, NY), as specified by the distributor. The total RNA isolated was quantified by spectrophotometry and the corresponding HIF-1α and 18S transcripts were amplified with 200 ng of cDNA by using semiquantitative end-point PCR. The conditions for the PCR reaction used to amplify the HIF-1α transcript (20 μL of final volume) were the following: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 3.5 mM MgCl₂ using 0.2 mM for each deoxyribonucleotide triphosphate and 1.0 μM for each primer, with 2.5 units of Taq DNA polymerase (BioTecMol, Mexico). Cycling conditions were the following: 95°C for 5 min and then 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. Primers used for HIF-1α detection (219 bp) were 5′–GCT TGG TGC TGA TTT GTG AA–3′ (forward) and 5′–GAT GGG TTT TGG TCA GAT GG –3′ (reverse).

Normalization was performed by using the 18S gene (154 bp), as previously reported (Mendoza-Garcés et al., 2013). Both assays produced a single PCR product at the molecular weight expected, as confirmed by using a 2% agarose gel stained with GelRed (Biotium). The corresponding bands were quantified by densitometry using the Vision Works LS software (UVP).

Statistical analysis was performed using the Graph Pad Prism 6 software (GraphPad Software). Experimental data are presented as the mean ± the SEM from 3 or more independent replicates. Two-way ANOVA tests were performed when analyzing 3 or more experimental conditions followed by the Tukey post hoc test. The Student t test was performed for each pair of experimental conditions. Statistical differences were considered with a P-value < 0.05.

RESULTS

Ewes’ Arterial Pressure

Arterial pressure is a classic physiological parameter used to study the normo-evolution of pregnancy. As depicted in Fig. 1, in both groups, the MAP was maintained between 75 and 125 mmHg during the last two-thirds of gestation. Except in wk 11 and 18, when the sheep with hybrid pregnancy presented decreased levels in MAP compared with normal pregnancy, there were no differences in this parameter between the 2 groups.

Proteinuria Levels throughout Sheep Pregnancies

To investigate renal endothelial dysfunction, urine protein content was evaluated during the last two-thirds of pregnancy in both the control and hybrid groups (Fig. 2). We did not find differences in urine protein content between normal and interspecies groups. However, a difference was detected only at the 10th week of pregnancy.

Offspring and Placental Weight at Hybrid Pregnancies

The hybrid offspring presented a mixture of the physical characteristics of both species. However, the phenotypic characteristics of lambs and placentas were evaluated at birth. Gestation time was extended in the hybrid group (151.5 ± 1.555 d) compared with the control group (146.7 ± 0.4944 d). Importantly, the hybrid group presented decreased offspring and placental weight at birth compared with the control group (2.42 ± 0.20 vs. 5.53 ± 0.28 kg and 0.337 ± 0.07 vs. 0.662 ± 0.04 kg, respectively; Fig. 3A and 3B). The placenta:fetus ratio was not different between both groups (P = 0.8507); however, there was a negative correlation between the fetal weight and placental weight of the hybrid group (R² = 0.9874, P = 0.0063), which was not observed in the control group (R² = 0.2433, P = 0.3201).

The birth proportion between sexes was of 50%, and there were no differences between sexes in any of the studied parameters (data not shown).

Cotyledonary Responses to Hybrid Pregnancies

Comparing the morphological characteristics of placentas from the hybrid pregnancies versus those from the normal pregnancies, the average cotyledonary weight (g) was smaller in the former group (17.8 ± 2.09 g vs. 35.0 ± 1.81 g; Fig. 4A), as was the average cotyledonary surface area (47.81 ± 5.18 cm² vs. 77.29 ± 3.25 cm²; Fig. 4B). On the other hand, we did not observe differences in the number of cotyledons between groups (data not shown).
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Cell-Free HIF-1α Gene Expression in Hybrid Pregnancies

It has been recently reported that gene expression studies can be performed in a cell-free RNA system (or extracellular RNA) that can be isolated from plasma to detect disease-associated biomarkers (Ashur-Fabian et al., 2012); therefore, we used this noninvasive strategy to study HIF-1α gene expression. Assessment of HIF-1α gene expression during the last two-thirds of gestation. As it can be observed, HIF-1α was above normal in the sheep with a hybrid pregnancy (compared with the control; Fig. 5). Particularly marked differences were detected at the 9th, 11th, and 14th week of pregnancy.

Progesterone Plasmatic Levels in Hybrid Pregnancies

Progesterone levels were measured in plasma from pregnant sheep in both groups. Interestingly, P4 levels in the hybrid group were decreased compared with the control group. There was a difference between the 2 groups in P4 levels at the 8th, 14th, and 16th to 21st weeks, and this was particularly pronounced as of the 16th week (Fig. 6).

VEGF Plasmatic Levels in Hybrid Pregnancies

By using ELISA, the VEGF level was measured in plasma taken from sheep with a hybrid pregnancy and from animals with a normal pregnancy (control), and the levels in the former group were greater from the 17th to 19th weeks (Fig. 7).

PIGF Plasmatic Levels in Hybrid Pregnancies

Regarding the level of PIGF, we observed no significant differences between the 2 groups during the first 20 wk of gestation (Fig. 8). However, there was a tendency to greater levels of this parameter in the hybrid versus control group as of the 17th week of gestation, which became significant in the 21st week.

DISCUSSION

Hybrids represent a strategy for the conservation of genetic characteristics in highly endangered species. However, hybrids involve a genetic admixture that can challenge the genetic integrity of wild and domestic populations. Hence, there is a continuous search for stable hybrids to avoid or diminish the loss of unique and essential genetic material (Santiago-Moreno et al., 2001; Ptak et al., 2002). Hybridization between wild and domestic bovine species occurs worldwide, either spontaneously or by design. For example, the well-known model based on crossing Bos taurus and Bos indicus (European and Asian domestic cattle, respectively) produces fertile hybrids characterized by an increase in heat tolerance and disease
resistance compared with the former species (Nijman et al., 2003; Verkaar et al., 2003). In contrast, other models of hybridization have not been as successful as the previous one. In the present study, we report the phenotypic and molecular characteristics of the F$_1$ population of hybrid pregnancies resulting from crossing O. canadensis mexicana with O. aries; the former is a unique Mexican endangered specie.

When comparing the characteristics of domestic sheep pregnancy with those of the hybrid pregnancy, we found that at the time of birth, the hybrid group showed small offspring and diminished placental weight, reduced fetal/placental growth, and a decrease in cotyledonary surface area. In addition, a severe chronic placental insufficiency was evidenced by a significant decrease in P4 production. Moreover, analysis of maternal plasma indicated that sheep with a hybrid pregnancy had endothelial dysfunction, characterized by an increase in VEGF, PlGF, and HIF-1$\alpha$ mRNA levels.

Despite there being no differences in the placenta:fetus weight ratios between the hybrid and control groups, a negative correlation between the fetal weight and placental weight of the hybrid group was observed, which suggests that restriction growth observed in this sheep interspecies pregnancy is proportional in both product and placental tissues. In contrast, we did not find a correlation between the fetal weight and placental weight of the control group. This relationship between fetal and placental weight has been previously reported and has been associated with the physiological exchange provided by the placental circulation, which, in turn, explains the proportional weight decrease observed in this study (Reynolds and Redmer, 1995, 2001; Reynolds et al., 2005a,b; Myatt, 2006).

It has been reported that the gestation time of big-horn sheep is 179 ± 6 d (Turner and Hansen, 1980; Dermachi, 2004) and 149 d for the domestic sheep (Pérez-Clariget and Porras, 2008). Interestingly, gestation time in the hybrid group was extended com-
pared with the control group and intermediate to both species, which suggests that a compensation process could be involved to complete the fetus growth process. Despite this extended gestation time described in the hybrid group, the fetus weight in this group was smaller when comparing with the control group, which suggest that this increase in gestation time was not enough to compensate the restriction growth observed in the hybrids, as reported in other models (Regnault et al., 2002; Morrison, 2008; Gourvas et al., 2012a,b. Unfortunately, there is no data available about birth weight in bighorn sheep to compare with the results presented in this study.

It is well known that nutrients from maternal blood reach fetal circulation through the placenta, which plays a major role in nutrition and fetal growth (Ahmed and Perkins, 2000; Wu et al., 2004; Reynolds et al., 2005a,b, 2010; Roland et al., 2012). Moreover, recent studies have shown differences in fetal size, placental and fetal growth, and postnatal survival between natural and assisted reproductive pregnancies (Grazul-Bilska et al., 2014; Kondapalli and Perales-Puchalt, 2013; Reynolds et al., 2014). The reduced size observed in both offspring and placenta as well as the decreased growth of hybrid offspring (compared with the control) are similar to those observed in fetal growth restriction syndrome in other animal models (Regnault et al., 2002, 2003; Reynolds et al., 2005a,b; Barry and Russell, 2008).

Interestingly, in a recent meta-analysis, it was determined that the severity of fetal intrauterine growth restriction (IUGR) caused by multifetal pregnancies was comparable to the effects caused by experimentally induced fetal growth inhibiting interventions. These interventions represent unfavorable conditions during pregnancy in sheep, such as long-term malnutrition, adolescent overfeeding, and embolization (Goettwine, 2013). These models have provided an increased understanding of the nature of fetal adaptations to IUGR, their long-term physiological consequences, and how to improve clinical management of IUGR pregnancies (Morrison, 2008; Barry and Russell, 2008). The current study of domestic sheep with a hybrid pregnancy represents a constraint model of IUGR syndrome.

In our model, we sought to explore possible systemic and renal endothelial dysfunction in ewes by assessing the blood pressure and protein urinary content during the last two-thirds of pregnancy. To our knowledge, this is the first report about urinary protein content during pregnancy in sheep, although no differences were found between groups during the 14-wk evaluation period and were similar to those previously reported for normal non-pregnant sheep (Mendoza-Garcés et al., 2013).

In relation to blood pressure, previous studies only report the measurement of the central blood pressure. In contrast, our study is the first report using an external noninvasive method. We observed that the MAP remained between 75 and 125 mmHg throughout the last two-thirds of gestation in both the hybrid and control groups. In contrast, Adams and McKinley (2009) have reported 70 mmHg in nonpregnant sheep. The greater values found herein are probably due to the pregnancy of the animals and the methodological approach used.

The fact that there were no differences in MAP between the hybrid and control group indicates the absence of systemic endothelial dysfunction in the sheep with a hybrid pregnancy. To further evaluate the endothelial function during pregnancy, we assessed different angiogenesis-related factors, including VEGF, PlGF, and HIF-
VEGF expression is found to be increased compared with α, which have been reported to be altered in pathological conditions (Reynolds et al., 2005a, b; Morrison, 2008).

Angiogenesis is a multifactorial process involving the formation of new vascular beds and is critical for the growth and development of normal tissue, including that of the placenta (Reynolds and Redmer, 2001; Borowicz et al., 2007; Reynolds et al., 2010). Among the numerous angiogenic factors required for normal placental development (e.g., for proliferation, vascularization, and cell migration of trophoblastic cells), the most important are VEGF, fibroblast growth factor, angiopoietin 1 and 2, and PIGF (Reynolds and Redmer, 2001; Regnault et al., 2003; Tammela et al., 2005; Borowicz et al., 2007). It has been reported that all of these factors are mainly regulated by HIF-1α (Bracken et al., 2003; Majmundar et al., 2010). Under normal conditions, these factors are expressed during the early stages of placental development and then decrease throughout pregnancy. Although it is known that they favor trophoblastic maturation and placenta formation, other roles of these factors have also been reported during pregnancy (Caniggia et al., 2000; Pringle et al., 2010).

Previous studies have reported that VEGF and its receptors are expressed on ovine placental and fetal membranes as well as on the endothelium of maternal blood vessels throughout gestation (Bogic et al., 2001; Reynolds et al., 2005a, b). In fact, VEGF has been associated with many endothelial functions such as survival, proliferation, and migration of endothelial cells (Borowicz et al., 2007; Reynolds et al., 2010). Moreover, in different pathologies during human pregnancy, including preeclampsia and fetal placental growth restriction, VEGF expression is found to be increased compared with normal pregnancies (Maynard et al., 2003; Levine et al., 2004; Maynard and Karumanchi, 2011). Indeed, Barut et al. (2010) reported a significant increase in VEGF expression during IUGR that displays a pattern similar to that observed in the present study. The increased VEGF levels found in the hybrid group, in the present study, were probably due to an increase in fetal growth and nutrient requirements at this period of fetal development.

Interestingly, Vonnahme et al. (2005) observed an increase in the level of serum VEGF during the last stage of pregnancy in multiple ovine gestations compared with a single ovine gestation. In the present study, this factor also showed a significant increase at the end of pregnancy in the hybrid versus the control group, despite hybrids gestation presenting diminished fetal size, offspring birth weight, and fetal development; therefore, this increase in VEGF supports the idea that it should be due to an endothelial dysfunction.

In addition to VEGF, an altered expression pattern has been reported for PIGF and its receptors in placental cotyledons and caruncles during placental growth on a model of fetal growth restriction. In fact, the authors suggested that this might contribute to IUGR (Regnault et al., 2002, 2003). The increase in VEGF and PIGF levels found in the hybrid group (from the 13th to 19th week and in the 20th week of gestation, respectively) was also associated with IUGR.

Another key molecule is HIF, an oxygen-regulated transcription factor, which plays a major role in angiogenesis during fetal and placental mammalian development (Caniggia et al., 2000; Semenza, 2001; Tal, 2012; Gourvas et al., 2012a, b). Song et al. (2008) reported that HIF-1α is expressed in the endometrium of pregnant and cyclic ewes as well as during peri-implantation blastocyst development. Borowicz et al. (2007) observed an increase in the levels of HIF-1α mRNA expression in caruncular and cotyledary sheep tissues from Day 50 to 110 of pregnancy, a period of time when blood vessels grow more rapidly, indicating a role of HIF1α in sheep placental angiogenesis; in the present study, we observed a similar pattern of plasmatic HIF-1α expression in the control group. In contrast, an increase and almost constant HIF-1α expression in the hybrid group vs. the control group was found at wk 9, 11, and 14 of gestation. This alteration in the expression of HIF-1α and its target proteins suggests a continuous state of hypoxia in the ewes with a hybrid pregnancy. It is known that HIF-1α is overexpressed in mammals with different pathologies involving uterine growth restriction, such as high altitude pregnancies, hemoglobinopathies, diabetes, and preeclampsia. Interestingly, it has been recently reported that the plasmatic cell-free HIF-1α mRNA levels are altered in preeclampsia and IUGR, which highlights HIF-1α expression levels as a marker of growth restriction and hypoxia (Ashur-Fabian et al., 2012).

Conceptus–endometrial interactions in ruminants are complex and involve strictly orchestrated temporal and spatial changes in endometrial gene expression (Spencer and Bazer, 2002; Spencer et al., 2004; Dorniak and Spencer, 2013; Ulbrich et al., 2013). Progesterone, “the pregnancy hormone,” is required for the establishment and maintenance of pregnancy in all mammals, specifically to support the secretory functions of the endometrium that sustain embryonic development, implantation, and placentation (Spencer et al., 2004, 2007; Bazer et al., 2009).

In a recent study by Bazer et al. (2015), the authors examined the development of ovine conceptus throughout gestation. This study included many biochemical, metabolic, and phenotypic parameters of the fetus and the ewe. The authors observed changes in plasmatic levels of steroid hormones during pregnancy, particularly P4. The level of the latter hormone changed according to the day of gestation, with a maximal value at Day 80 of pregnancy (Parraguez et al., 2013; Bazer et al., 2015). The pattern of
the level of P4 reported in the aforementioned study was similar to that found in the control group of the current study. On the other hand, significantly reduced levels of P4 were observed in the hybrid versus the control group since wk 14, therefore indicating placental deficiency (Moffatt et al., 1987; Spencer and Bazer, 2002).

The overall results of the current contribution indicate that the interspecies crossing between Mexican Ovis canadensis and O. aries produces IUGR, mainly due to an alteration in endothelial function and decreased P4 levels leading to chronic placental dysfunction. Further studies are needed to overcome these placental alterations and thus improve offspring viability, possibly through P4 treatment and special nutritional conditions. Such treatments may provide the opportunity for future genomic evaluations of these hybrids.

LITERATURE CITED


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