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Steroidogenesis in sheep pregnancy with intrauterine growth retardation by high-altitude hypoxia: effects of maternal altitudinal status and antioxidant treatment

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Abstract. Sheep pregnancy in high-altitude environments frequently involves hypoxia and oxidative stress and causes intrauterine growth retardation. The adverse effects of altitude on fetal growth can be prevented by the administration of antioxidant vitamins, but the mechanisms responsible are not well known. The maintenance of a viable pregnancy depends largely on adequate placental steroidogenesis, especially in the last two-thirds of pregnancy. Thus, in the present study we evaluated the effect of antioxidant vitamins (C and E) on plasma concentrations of progesterone and 17β-oestradiol during the last two-thirds of high-altitude pregnancies in ewes both native and naïve to the high-altitude environment. In addition, pregnancy outcomes were evaluated by determining the bodyweight of newborn lambs. Sex steroid patterns differed between ewes with and without vitamin supplementation. Concentrations of plasma progesterone and 17β-oestradiol were significantly higher in the supplemented groups from approximately 40 days before parturition until near term. Newborn weights were significantly lower in animals not adapted to the higher altitude, and vitamin supplementation prevented this decrease. In conclusion, the administration of antioxidant vitamins in the present study enhanced placental steroidogenesis, thus favouring fetal development in pregnancies developing at high altitudes.

Additional keywords: progesterone, 17β-estradiol, vitamins C and E.

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Introduction

Fetal viability and development, both in humans and animal species, is dependent on an adequate placental supply of oxygen and nutrients. Hypoxia and undernutrition may cause death of the conceptus or deficiencies in its growth, leading to intrauterine growth retardation (IUGR), as well as reduced birthweight and postnatal growth (Wu *et al.* 2006).

Fetal hypoxia has been observed in pregnancies complicated by pre-existing maternal illness, pre-eclampsia, cord compression, smoking, pollution, haemoglobinopathy and aberrant placental development causing placental insufficiency (Baschat 2004; Patterson and Zhang 2010). Recent reports also indicate that fetal hypoxia appears to be a major cause of stillbirths in diabetic pregnancies (Teramo 2010). However, the highest incidence of fetal hypoxia occurs in pregnancies at high altitudes (Moore 1990). Epidemiological data show that high-altitude hypoxia increases the risk of IUGR, premature birth, low birthweight and infant mortality, especially in individuals exposed to high-altitude conditions for a short period of time (Jensen and Moore 1997; Moore *et al.* 2001).

Studies into the features of pregnancies under conditions of high-altitude hypoxia (hypobaric hypoxia) are important in their own right (~140 million people inhabit altitudes >2500 m above sea level (a.s.l.) and an additional 40 million visit these regions yearly; Moore *et al.* 1998), but they are also important for the potential implications in the assessment of pregnancies with placental insufficiency in populations living at sea level. However, data from human pregnancies at high altitudes are biased because of usually concurrent economic impoverishment and malnutrition. Information is also limited because of interventional research cannot be conducted because of ethical issues; thus, investigations need to be conducted in animal models. The sheep is especially interesting for pregnancy studies owing to its similarities to humans, including singleton pregnancies and a similar developmental trajectory (Barry and Anthony 2008; Poore *et al.* 2010). Working with this species allows for the control of environmental conditions, including the nutrition of these animals during the study period, to avoid confounding effects.

Furthermore, studies on high-altitude ovine pregnancies have a dual purpose. In addition to being a recognised biomedical model, the sheep is a key economic resource for highland native farmers from developing and transition regions, including those living in the Andean and Qinghai–Tibetan plateaus; approximately 25 million rural people depend directly on sheep breeding (Huddleston and Ataman 2003). The reproductive features of the ovine, like in humans, are affected by altitude and are mainly characterised by lower fertility (Lanino 1977; De Carolis 1987), a higher incidence of asymmetric IUGR (Parraguez *et al.* 2005) and lower birthweight and postnatal growth (Parraguez *et al.* 2004). These features are accentuated in animals newly arriving to the high-altitude environment (Parraguez *et al.* 2004, 2006*a*), as is also the case in humans (Moore *et al.* 2001, 2004; Hartinger *et al.* 2006), and this is a major limitation to improving animal production at high altitude by introducing selected animals from the lowlands.

In a previous study, we have shown that pregnancy at high altitudes is concurrent with remarkable adaptive changes in the placenta, including increased weight, placentome size and vascularisation (Parraguez *et al.* 2006*a*). It is currently well recognised that all the processes of neovascularisation and angiogenesis are regulated by steroid hormones in mammals (Rogers *et al.* 2009). The placenta has an active role in the secretion of its own steroids (Pepe and Albrecht 1995) and, specifically in sheep, pregnancy is supported by placental steroidogenesis from Days 50–60 onwards (Bassett *et al.* 1969). Thus, the first aim of the current study was to determine the effect of altitudinal status on the secretion of progesterone and 17β-oestradiol from Day 70 of pregnancy to delivery.

In recent studies, we have demonstrated that the deleterious effects of oxidative status induced by high altitude may be prevented by daily administration of the antioxidant vitamins C and E, improving placental function and increasing newborn weight and viability (Parraguez *et al.* 2011). Thus, the second aim of the present study was to assess, in sheep both native and naïve to high altitudes, the effects of such treatment from Day 20 of pregnancy onwards on the secretion of progesterone and 17β-oestradiol during the second half of gestation.

Materials and methods

Animals and management

The present study was performed at the International Centre for Andean Studies (INCAS; University of Chile, Arica–Parinacota region, Chile), located 3589 m a.s.l., where the barometric pressure is approximately 667 hPa. Experimental procedures were developed in accordance with the International Guiding Principles for Biomedical Research Involving Animals (Council for International Organisation of Medical Sciences, World Health Organisation, http://cioms.ch/publications/guidelines/1985_texts_of_guidelines.htm, accessed on May 2007) and were approved by the Bioethics Review Committee of the Faculty of Veterinary and Animal Sciences, University of Chile, as well as the Bioethics Advisory Committee of the Chilean National Commission for Scientific and Technological Research (CONICYT).

Singleton pregnant Creole ewes (Chilean mix breed developed from Churra and Manchega Spanish breeds; $n = 24$) with a history of normal pregnancies and deliveries were used. Twelve sheep were native to high altitudes and were descendants of sheep introduced to the Andean high plateau by Spanish settlers almost 500 years ago (Group HH). The other 12 sheep were native to the lowlands (Valle de Lluta, Arica–Parinacota Region, Chile; 500 m a.s.l.; barometric pressure 990 hPa), with similar phenotypes and bodyweights to the HH ewes (Group LH). To prevent the reduction in the fertility of LH sheep when taken to high altitudes for mating, as reported previously (Parraguez *et al.* 2006*b*), both HH and LH sheep were mated with males of proven fertility in their respective places of origin. Twenty days after mating, pregnancy diagnosis was performed by ultrasound examination and LH ewes were taken to the INCAS experimental station where they joined the HH ewes. All sheep were then kept together and were provided daily with alfalfa hay (~2 kg day^{-1} per animal; dry matter 88.9%, metabolisable energy 10.6 MJ kg^{-1} dry matter; crude protein 14.5%) and fresh water *ad libitum*. The food supply was calculated to satisfy daily ovine requirements in late gestation (National Research Council, 1985).

To evaluate the effects of vitamin supplementation on progesterone and 17β-oestradiol concentrations, half the animals from each group were randomly selected, separated and daily treated with antioxidant vitamins from Day 20 after mating until the end of the experiment. To this end, every day early in the morning, 0.5 kg alfalfa supplemented with 500 mg vitamin C and 350 IU vitamin E was administered to each sheep in an individual feeding trough. After consumption of this ration, the total daily amount of alfalfa was provided. Thus, there were four groups in total in the present study ($n = 6$ in each group): (1) HH ewes, native to and developing pregnancy at high altitude; (2) HHV ewes, vitamin-supplement ewes native to and developing pregnancy at high altitude; (3) LH ewes, native to sea level but developing pregnancy at high altitudes; and (4) LHV ewes, vitamin-supplement ewes native to sea level but developing pregnancy at high altitudes.

Sampling and measurements

At Day 60 of gestation, all ewes were anaesthetised with 20 mg kg^{-1}, i.m., ketamine (Ketamil; Troy Laboratories, Smithfield, NSW, Australia) for the surgical placement of a polyvinyl catheter (2.5 mm internal diameter; Tygon; Saint Gobain Performance Plastics, Akron, OH, USA) in the left femoral artery and vein. Once installed, the catheters were filled with heparinised saline (1000 IU mL^{-1}) to prevent clot formation. Catheters were then passed through the subcutaneous tissue to the left flank of the ewe, where they were exteriorised and

Table 1. Blood gases in pregnant sheep at high altitude and the effects of vitamin C and E supplementation
Data are the mean ± s.e.m. Within a column, different superscript letters indicate significant differences ($P < 0.05$). HH, ewes native to and developing pregnancy at high altitude; HHV, vitamin-supplement ewes native to and developing pregnancy at high altitude; LH, ewes native to sea level but developing pregnancy at high altitudes; LHV, vitamin-supplement ewes native to sea level but developing pregnancy at high altitudes; P_aO_2, arterial partial pressure of oxygen; P_aCO_2, arterial partial pressure of carbon dioxide; Hct, haematocrit; Hb, haemoglobin; SatHb, Hb saturation with oxygen

	P_aO_2 (mmHg)	P_aCO_2 (mmHg)	Hct (%)	Hb (mg dL^{-1})	SatHb (%)	pH
HH	53.0 ± 2.1^{ab}	21.8 ± 2.4	32.2 ± 1.2	12.5 ± 0.5^{b}	82.6 ± 1.8^{b}	7.50 ± 0.02
HHV	60.4 ± 5.6^{c}	25.8 ± 2.9	33.3 ± 2.1	12.9 ± 1.1^{ab}	85.9 ± 3.9^{bc}	7.48 ± 0.05
LH	49.5 ± 2.3^{a}	25.0 ± 2.6	35.0 ± 0.7	14.3 ± 0.4^{a}	77.5 ± 3.4^{a}	7.48 ± 0.01
LHV	58.8 ± 4.1^{bc}	25.4 ± 3.0	33.2 ± 3.1	13.4 ± 1.4^{ab}	85.5 ± 1.4^{bc}	7.47 ± 0.07

placed in a canvas pocket attached to the skin. Arterial and venous blood samples were collected once a week, from 10 days after surgery until the end of gestation.

Arterial blood samples were used to assess the oxygenation status of the animals. Oxygenation status was determined on the basis of P_aO_2, P_aCO_2, haematocrit (Hct), haemoglobin (Hb) concentrations, Hb saturation by oxygen (SatHb) and pH values. These measurements were performed using an IL Synthesis 25 gas analyser (Instrumentation Laboratory, Bedford, MA, USA), calibrated to local atmospheric pressure and ovine body temperature.

Progesterone and 17β-oestradiol concentrations were determined in venous plasma. Blood samples were obtained in heparinised syringes and centrifuged at 800*g* for 5 min. The plasma was stored at −20°C until use.

Progesterone concentrations were analysed using a solid-phase radioimmunoassay (RIA) specific for this steroid with reagents and techniques provided by DPC Coat-a-Count (Diagnostic Corporation, Los Angeles, CA, USA). Samples were measured in duplicate. The sensitivity of the assay was 0.1 nM (0.031 ng mL^{-1}). The intra- and interassay CV were 4.9% and 8.5%, respectively.

Concentrations of 17β-oestradiol were measured using a double-antibody RIA without prior extraction using the reagents and techniques provided by DPC (Diagnostic Corporation). Analyses were performed in duplicate. The sensitivity of the assay was 5.18 pM (1.4 pg mL^{-1}). The calculated intra- and interassay CV were 3.5% and 4.9%, respectively.

At birth, newborn lambs were weighed using an electronic scale (Simplex 150; Shanghai Yaohua Weighing System, Shanghai, China).

Statistical analysis

Data collected for hormone concentrations, oxygenation status and the weight of newborn lambs were analysed using the general linear model (GLM) procedure (SAS Institute, Cary, NC, USA). The statistical model included two crossed factors, altitudinal status and vitamin supplementation, as well as their interactions. For a best hormone profile comparison, avoiding the effect of small differences in length of gestation, hormone data were arranged retrospectively from the day of parturition. The time of pregnancy, a continuous variable, was introduced into the model as a covariable and then analysis of covariance was performed. $P < 0.05$ was considered significant. When significant differences were found, Duncan's test was performed to establish where the differences were. If between-group differences were detected, the data were compared according to different times of gestation to determine the origin of such differences. All results are expressed as the mean ± s.e.m.

Results

The results of assessments of arterial blood gases and other variables related to oxygen transport are given in Table 1. There were no differences in P_aCO_2, Hct and pH among the groups, but Hb was significantly higher in LH ewes, it was at an intermediate level in both vitamin-treated groups (HHV and LHV) and was significantly lower in HH ewes ($P < 0.05$). There was a significant improvement in P_aO_2 in the vitamin-treated groups compared with their untreated counterparts ($P < 0.05$). The highest values for SatHb were obtained in the two vitamin-treated groups and although the difference between the LH and LHV groups was significant ($P < 0.05$), that between the HH and HHV groups was not.

Gestational length in the HH, LH, HHV and LHV groups was 152.9 ± 3.6, 148.5 ± 5.3, 151.9 ± 4.2 and 152.6 ± 6.8 days, respectively ($P > 0.05$).

Altitudinal status had a significant effect on the bodyweight of newborn lambs. The lambs in the HH group were heavier than those in the LH group (3.7 ± 0.3 vs 3.1 ± 0.5 kg, respectively; $P < 0.01$). Antioxidant therapy increased the weight of lambs in the LHV group only (3.7 ± 0.5 kg; $P = 0.01$). Although the highest bodyweight was seen for lambs in the HHV group (4.0 ± 0.5 kg), it did not differ significantly from that of lambs in the HH and LHV groups ($P > 0.05$). Thus, when comparing all the groups, the administration of antioxidant vitamins increased birthweight such that the supplemented LHV lambs were of similar weight to those from mothers native to the high-altitude environment.

Retrospective analysis of steroid secretion showed significant effects of both altitudinal status and antioxidant therapy on progesterone secretion. Mean plasma progesterone concentrations during pregnancy were lower in HH compared with LH ewes (48.1 ± 6.7 vs 68.8 ± 8.8 nmol L^{-1}, respectively; $P < 0.001$). Treatment with antioxidant vitamins significantly increased mean plasma progesterone concentrations in both

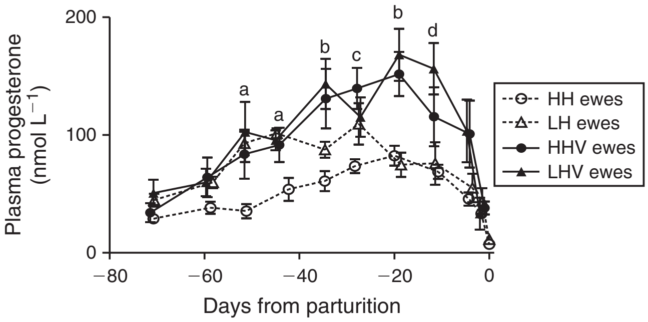

Fig. 1. Plasma progesterone concentrations in pregnant ewes during gestation. HH, ewes native to and developing pregnancy at high altitude; HHV, vitamin-supplement ewes native to and developing pregnancy at high altitude; LH, ewes native to sea level but developing pregnancy at high altitudes; and LHV, vitamin-supplement ewes native to sea level but developing pregnancy at high altitudes. Data are the mean ± s.e.m. progesterone concentrations in blood samples that were obtained weekly. Day 0 is the day of delivery. [a]$P < 0.05$ for HH ewes compared with all other groups at the same time point; [b]$P < 0.05$ for HH and LH ewes compared with HHV and LHV ewes at the same time point; [c]$P < 0.05$ for HH compared with HHV ewes at the same time point; [d]$P < 0.05$ for HH and LH compared with LHV ewes at the same time point.

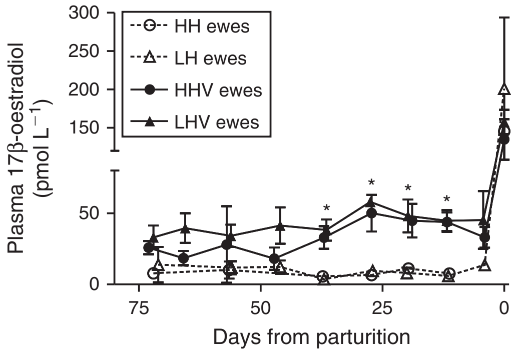

Fig. 2. Plasma 17β-oestradiol concentrations in pregnant ewes during gestation. HH, ewes native to and developing pregnancy at high altitude; HHV, vitamin-supplement ewes native to and developing pregnancy at high altitude; LH, ewes native to sea level but developing pregnancy at high altitudes; and LHV, vitamin-supplement ewes native to sea level but developing pregnancy at high altitudes. Data are the mean ± s.e.m. 17β-oestradiol concentrations in blood samples that were obtained weekly. Day 0 is the day of delivery. *$P < 0.05$ compared with ewes not receiving vitamin supplementation at the same time point.

the HHV and LHV ewes (95.0 ± 12.9 vs 102.8 ± 14.3 nmol L^{-1}, respectively; $P < 0.001$). Moreover, there was a significant interaction between altitudinal status and vitamin treatment ($P < 0.05$).

The profile of plasma progesterone concentrations obtained during the gestational period under investigation was also affected by altitudinal status (Fig. 1). The HH group showed a gradual increase in progesterone concentrations until Day 20 before delivery, when concentrations peaked (82.5 ± 9.2 nmol L^{-1}); after this point, the progesterone concentration decreased until delivery. In contrast, the LH group exhibited a higher rate of increase of plasma progesterone concentrations, with a peak of 100.5 ± 19.2 nmol L^{-1} occurring on Day 27 before delivery. The progesterone concentration profiles in HHV and LHV were similar, with the highest concentrations seen on Day 20 before lambing (151.8 ± 18.7 and 168.2 ± 22.1 nmol L^{-1}, respectively). Covariance analysis indicated that these concentration–time curves were significantly different ($P < 0.001$), with progesterone concentrations in the HH and LH groups significantly different from concentrations in the other groups at almost every time point of gestation ($P < 0.05$).

There were no significant effects of either altitudinal status or antioxidant therapy on mean 17β-oestradiol concentrations throughout pregnancy. Assessment of the interaction between both variables showed that the administration of vitamin to LHV animals was associated with a higher plasma 17β-oestradiol concentrations than in the HHV group ($P < 0.05$). However, analysis of mean 17β-oestradiol concentrations, after discarding the values obtained at delivery, indicated no effect of altitudinal status (17β-oestradiol concentrations of 9.6 ± 1.6 and 9.8 ± 1.3 pmol L^{-1} in the HH and LH groups, respectively; $P > 0.05$) but a significant effect of antioxidant treatment, because 17β-oestradiol concentrations in the HHV and LHV groups (32.8 ± 3.8 and 42.5 ± 2.6 pmol L^{-1}, respectively) were significantly higher than concentrations in their untreated counterparts ($P < 0.001$).

The lack of significant differences in mean 17β-oestradiol concentrations during the last half of pregnancy may be due to a proper secretion profile of the hormone, as shown in Fig. 2. In all groups, plasma 17β-oestradiol concentrations were maintained with little variation until the time of delivery, when concentrations increased sharply. The mean 17β-oestradiol concentrations at delivery were similar in the HH, HHV, LH and LHV groups (145.5 ± 38.9, 134.9 ± 26.3, 201.0 ± 92.9 and 157.7 ± 15.8 pmol L^{-1}, respectively; $P > 0.05$). However, plasma 17β-oestradiol concentrations at delivery were several-fold higher than in the previous period, thus masking any differences observed throughout the gestational period. The slopes of the 17β-oestradiol curves were found to be different ($P < 0.05$); therefore, comparisons of the different gestation time points were made. It was found that the LHV and HHV groups had higher 17β-oestradiol concentrations than their untreated counterparts at four time points between Days 37 and 11 before delivery ($P < 0.05$).

Discussion

Based on the results of both blood gas composition and variables related to O_2 and CO_2 transport, the ewes in the present study clearly showed hypoxia in a manner similar to sheep described in a previous study living under the same conditions (Parraguez *et al.* 2006*a*). The ewes that were newcomers to the high-altitude environment had increased Hb levels, which indicates the development of acclimation mechanisms, but an even higher degree of hypoxia than ewes native to high altitudes; the percentage SatHb was significantly lower in the former group. Thereafter, the exposure of lowland-native ewes to hypobaric hypoxia during pregnancy induced the phenomenon of IUGR and reduced birthweight. These results are consistent with data reported previously in animals under similar (Jacobs *et al.* 1988) or the same (Parraguez *et al.* 2005) environmental conditions and confirm the usefulness of our model for studying the effects of high-altitude hypoxia on pregnancy.

The results of the present study also confirm that the administration of antioxidant vitamins has a positive effect on oxygen availability in the mother and, presumably, her fetus, diminishing the effects of hypoxia and oxidative stress on IUGR. The route of administration and vitamin doses used in the present study improved the parameters indicative of acclimatisation to high-altitude hypoxia, as shown in a previous study under the same conditions (Parraguez *et al.* 2011). Confirming the results of the previous study, the administration of antioxidants to ewes born at sea level during high-altitude pregnancy (i.e. the LHV group) resulted in a significant increase in the weight of their newborns. In fact, the weight of newborn lambs in the LHV groups was similar to that of lambs born to sheep native to high altitudes, either treated or untreated with antioxidants (HHV and HH groups, respectively). Furthermore, the administration of vitamins to HHV sheep resulted in a tendency for an increase in the weight of newborn lambs, although the difference failed to reach statistical significance.

We can hypothesise that the observed changes in $P_{a}O_2$ and SatHb in response to antioxidant therapy may improve birthweight by providing better oxygenation to the fetal–placental unit. This effect may be increased by a rightward shift of the curve of dissociation of oxygen from Hb, as has been demonstrated under conditions of hypobaric hypoxia (West 2002). The oxygen supply to the conceptus would also be improved in animals treated with vitamin C by a direct positive effect of this compound on ovine umbilical blood flow (Thakor *et al.* 2010) and by the effects on the endocrine environment discussed below, because steroids act on placental blood flow (Rosenfeld *et al.* 1976).

The results of the present study indicate that altitudinal status (sheep native to a higher altitude for several generations vs newcomers) and exposure to hypoxia have significant effects on steroidogenic function. This finding is of note because information in the literature on the effects of hypobaric hypoxia and/or oxidative stress induced by hypoxia on the synthesis or secretion of reproductive steroids is virtually non-existent.

The pattern of changes in plasma progesterone concentrations throughout pregnancy in sheep native to a high-altitude environment (i.e. the HH group) was consistent with previous reports for the ovine (Bassett *et al.* 1969; Wallace *et al.* 1997; Rutter and Russo 2002). This pattern is characterised by a slow increase in concentrations that reach peak values between Days 130 and 140 of gestation; thereafter, progesterone levels decline rapidly until delivery. However, plasma progesterone concentrations of the ewes acclimatised to hypoxia were higher than those reported for lowland ewes (Bassett *et al.* 1969; Rutter and Russo 2002). This finding is in agreement with data reported in women who developed their pregnancy at 3100 m a.s.l. in whom plasma progesterone concentrations were higher than those in women who developed their pregnancy at an altitude of 1600 m a.s.l. (Zamudio *et al.* 1994). Indigenous non-pregnant women residing at a high altitude have been reported to have greater plasma progesterone concentrations during the luteal phase of the menstrual cycle compared with women living at sea level (León-Velarde *et al.* 2001).

Sheep naïve to high-altitude environments (i.e. the LH group) exhibited a more rapid increase in progesterone concentrations, reaching maximum values earlier than the HH group. Moreover, progesterone concentrations in the LH sheep were significantly higher than in HH ewes, especially between Days 50 and 30 before delivery. To our knowledge, there are no data in the literature that explain these differences; however, we hypothesise that these differences are part of the acute response to exposure to a hypoxic environment. Previous studies have indicated an increase in plasma progesterone concentrations in pregnant women with pre-eclampsia, a reproductive pathology characterised by placental hypoxia (Salas *et al.* 2006). In this disease, placental hypoxia decreases cytochrome P450 aromatase activity, which decreases the conversion of progesterone to oestrogen and could lead to increased plasma progesterone concentrations (Jiang *et al.* 2000). In addition, Walsh (1988) noted that pre-eclamptic placentas have more pregnenolone sulfatase activity; thus, pregnenolone sulfate is more actively converted to progesterone. Although these results come from other experimental models, the results are consistent and may help explain the results obtained in our sheep model.

Administration of antioxidant vitamins significantly increased plasma concentrations of progesterone. There is no previous information on the effects of oxidative status on plasma progesterone concentrations during pregnancy in any animal species. However, there are data from *in vitro* and *in vivo* studies of reproductive tissues. *In vitro*, an elegant study in different human choriocarcinoma cell lines clearly demonstrated that ascorbic acid (vitamin C) increases progesterone and oestradiol synthesis and secretion in a dose-dependent manner. This effect was mediated by enhanced expression of the steroidogenic enzymes P450 cholesterol side-chain cleavage, 3β-hydroxysteroid dehydrogenase type 1 and aromatase (Wu *et al.* 2008). In that study, α-tochopherol (vitamin E) showed a similar tendency (although the differences failed to reach statistical significance), suggesting an important participation of antioxidant activity in the steroidogenic response in these placenta-derived cells. These data are in agreement with those of a previous granulosa cell culture study, which also showed that ascorbic acid stimulates the production of progesterone (Byrd *et al.* 1993). *In vivo*, it has been established in women that the pathology of luteal deficiency (low plasma progesterone concentrations during the luteal phase of the menstrual cycle, usually leading to subfertility or infertility) is associated with the presence of oxidative stress. The administration of vitamin C to women affected by this disease significantly increases plasma progesterone and fertility (Henmi *et al.* 2003). In addition, vitamin E has been shown to be effective in the treatment of mammary dysplasia, a pathology that also presents with decreased plasma progesterone concentrations (London *et al.* 1981). Finally, results from a study in cows demonstrate a significant positive correlation between ascorbic acid and progesterone concentrations in follicular cyst fluid (Haliloglu *et al.* 2008). Overall, this body of evidence suggests an important role for vitamins C and E in the stimulation of progesterone synthesis and secretion.

Plasma 17β-oestradiol concentrations throughout pregnancy in the sheep are low and rise only 1–4 days before delivery (Challis 1971; Rutter and Russo 2002; Nasar and Rahman 2006). The observations in the present study in animals native or naïve to high-altitude environments and either treated or untreated with vitamin supplementation are consistent with this general pattern. As is the case for progesterone, there is little information in the literature regarding the effects of hypobaric hypoxia and/or hypoxia-induced oxidative stress on oestrogen concentrations in animals or humans. High altitude-native women developing gestation at a high altitude have decreased plasma oestradiol concentrations from Week 30 of gestation onwards compared with women developing pregnancy at a low altitude (Zamudio *et al.* 1994). This effect may be explained by a decrease in aromatase activity when the partial pressure of oxygen is decreased, as described for trophoblast cells in culture (Jiang *et al.* 2000). Owing to the low oestrogen concentrations in the peripheral blood of sheep, it is not possible to conclude that animals gestating at high altitudes have lower 17β-oestradiol concentrations than those reported for sheep gestating at sea level.

However, our results show a clear and significant increase in plasma oestradiol concentrations during the last 40 days of pregnancy in sheep treated with antioxidant vitamins. Again, previous data are scarce, although there are some data from the previously mentioned *in vitro* and *in vivo* studies on the action of these vitamin on reproductive tissues. The *in vitro* study with human choriocarcinoma cells showed that vitamin C and E supplementation enhanced the production of oestradiol in a dose-dependent manner (Wu *et al.* 2008). *In vivo*, women with mammary dysplasia treated with vitamin E for two consecutive menstrual cycles showed a tendency for increased plasma oestradiol concentrations during the luteal phase of the cycle (London *et al.* 1981). In subfertile women with luteal deficiencies, significantly increased plasma oestradiol concentrations, above those observed in the normal control group, were observed after daily administration of 750 mg vitamin C (Henmi *et al.* 2003). These results support the improvement of steroidogenic activity of the placenta reported in the present study.

In conclusion, the administration of antioxidant vitamins during the entire period of pregnancy in sheep kept at high altitudes (high altitude native or naïve) increased placental sex steroidogenesis. Furthermore, vitamin treatment of high altitude-naïve sheep resulted in improvements in the oxygen transport in maternal blood and increased the birth weight of lambs; a similar tendency was observed for sheep native to the high altitude, but the differences failed to reach statistical significance. These antioxidant effects may allow for an improvement in the lambs' postnatal survival and growth, thus also improving sheep production at high altitudes. The mechanisms by which sex steroid production is increased by vitamin C and E supplementation require clarification in future studies.

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