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Effects of antioxidant vitamins on newborn and placental traits in gestations at high altitude: comparative study in high and low altitude native sheep

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Abstract. The present study evaluated the hypothesis that the effects of hypoxia on sheep pregnancies at high altitude (HA) are mediated by oxidative stress and that antioxidant vitamins may prevent these effects. Both HA native and newcomer ewes were maintained at an altitude of 3589 m during mating and pregnancy. Control low altitude (LA) native ewes were maintained at sea level. Half of each group received daily oral supplements of vitamins C (500 mg) and E (350 IU) during mating and gestation. Near term, maternal plasma vitamin levels and oxidative stress biomarkers were measured. At delivery, lambs were weighed and measured, and placentas were recovered for macroscopic and microscopic evaluation. Vitamin concentrations in supplemented ewes were two- or threefold greater than in non-supplemented ewes. Plasma carbonyls and malondialdehyde in non-supplemented ewes were consistent with a state of oxidative stress, which was prevented by vitamin supplementation. Vitamin supplementation increased lamb birthweight and cotyledon number in both HA native and newcomer ewes, although placental weight and cotyledon surface were diminished. Placentas from vitamin-supplemented HA ewes were similar to those from ewes at sea level, making these placental traits (weight, number and diameter of cotyledons) similar to those from ewes at sea level. Vitamin supplementation had no effect on LA pregnancies. In conclusion, supplementation with vitamins C and E during pregnancy at HA prevents oxidative stress, improving pregnancy outcomes.

Additional keywords: hypoxia, ovine, oxidative stress, pregnancy.

Introduction

At high altitude (HA), hypobaric hypoxia has significant effects on several characteristics of the ovine reproductive process. Recently, we demonstrated substantial differences in the magnitude of the effects of HA on pregnancy and newborn traits between ewes that have lived at HA for several generations, those native to low altitude (LA) that had been exposed to HA only from the beginning of the pregnancy and LA ewes that gestated at sea level. These effects included lower birthweight at HA (by ~23% in HA native sheep and 29% in LA native sheep) (Parraguez *et al.* 2005), increased cotyledon diameter in both HA and LA native ewes (by 70 and 26%, respectively), increased placental weight (by 41%) and a reduction in the number of cotyledons (by 25%) in HA native ewes, and tendency for increased placental weight (~8%) and a reduction in the number of cotyledons (~4%) in LA native ewes. In addition, the cotyledon–caruncle contact surface area was increased by 128 and 36% in HA and LA native ewes, respectively, whereas the cotyledon surface area occupied by the vasculature was increased by 135 and 75% in HA and LA native ewes, respectively (Parraguez *et al.* 2006).

Human pregnancy is also affected by HA, with low birthweight one of the most commonly observed effects (Yip 1987; Jensen and Moore 1997; Mortola *et al.* 2000; Giussani *et al.* 2001; Keyes *et al.* 2003; Moore *et al.* 2004; Hartinger *et al.* 2006). In a classic study performed in Colorado (USA), it was established that birthweight decreases by 102 g per 1000 m altitude and that the percentage of newborns with low birthweight increases by 54% at altitudes between 2744 and 3350 m above sea level (a.s.l.; Jensen and Moore 1997). Pregnancies at 4300 m a.s.l. show the effects of altitude on fetal growth from 25 weeks gestation onwards; 21% of fetuses had an estimated

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bodyweight below the fifth percentile of the population at sea level, and birthweight was 12% less than birthweight at sea level (Krampl *et al.* 2000). The incidence of low birthweight at 3600 m a.s.l. was almost threefold greater than that observed at LA (Keyes *et al.* 2003). Human pregnancy at HA is also characterised by increased presentation of asymmetrical fetal growth, fetal distress, preterm labour, premature rupture of the membranes, hypertension, pre-eclampsia and newborn respiratory distress (Krampl *et al.* 2000; Giussani *et al.* 2001; Keyes *et al.* 2003; Moore *et al.* 2004). Consistent with our observations in sheep, babies from mothers who were long-term residents at HA exhibited only 30–70% of the birthweight reduction observed in those babies from mothers who had recently arrived in an HA environment (Moore *et al.* 2001, 2004; Hartinger *et al.* 2006). Several studies in pregnant women at HA have shown changes in placental morphology and, although human and ovine placentas differ in structure and development, an increase in villous vascularisation and placental weight appears to be a common feature, at least in HA native populations (Zamudio 2003). However, a recent study in HA newcomer pregnant women at different altitudes reported a conserved vascularity and a reduction in placental weight (Tissot van Patot *et al.* 2009).

Exposure to a natural or artificial hypobaric hypoxic environment constitutes a physiological insult that leads to increased cellular production of reactive oxygen species (ROS), which may result in a state of oxidative stress (Joanny *et al.* 2001; Møller *et al.* 2001; Askew 2002; Magalhães *et al.* 2004, 2005). Emerging evidence suggests a close association between acute mountain sickness and oxidative stress (Bailey *et al.* 2001; Araneda *et al.* 2005). Furthermore, although pregnancy could be defined as a state of oxidative stress (Myatt and Cui 2004), pathological pregnancies, such as those complicated by pre-eclampsia, present a several-fold increase in oxidative stress biomarkers (Khalil and Granger 2002; Myatt and Cui 2004); the incidence of this pathology is approximately fivefold higher at HA than at LA (Palmer *et al.* 1999; Keyes *et al.* 2003). Antioxidant vitamin therapy has been shown to reduce hypoxia-induced oxidative damage (Ilavazhagan *et al.* 2001; Magalhães *et al.* 2005) and help prevent acute mountain sickness (Bailey and Davies 2001) and, although there is no consensus on this regard, there is considerable evidence showing it to be beneficial in preventing pre-eclampsia (Chappell *et al.* 1999, 2002; Rodrigo *et al.* 2005; Rumiris *et al.* 2006).

In the present study, we explored the hypothesis that the effects of HA hypobaric hypoxia on sheep pregnancies are mediated by oxidative stress by examining the ability of antioxidant vitamin therapy to improve placental characteristics and newborn bodyweight in HA and LA native sheep.

Materials and methods

The present study was performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals (Council for International Organisation of Medical Sciences, World Health Organization) and was approved by the Bioethics Review Committee of the Faculty of Veterinary Sciences, University of Chile, as well as by the Bioethics Committee of Chile's National Agency for Scientific Research (Comisión Nacional de Investigación Científica y Tecnológica).

Animal management

Forty time-mated (known gestational age) singleton pregnant Creole ewes (Chilean mix breed developed from the Churra and Manchega Spanish breeds), which had a history of two previous pregnancies and normal parturitions, were used in the HA experiment. Twenty ewes were HA native (3500–4000 m a.s.l.). These animals were descendants of the sheep herds introduced to the HA area by the Spaniard settlers and have thus been adapted to hypobaric hypoxia for over 500 generations. The other 20 ewes were LA native (<500 m a.s.l.). Thirty days before mating, the LA ewes were taken to our experimental station at HA (International Center for Andean Studies (INCAS), University of Chile; 3589 m a.s.l., barometric pressure (bP) = 667 kPa), where they joined the HA group. Animals were provided with alfalfa hay daily ($\sim$2 kg day^{-1}; dry matter (DM) = 90.2%, metabolisable enegery (ME) = 9.9 MJ kg^{-1}, crude protein (CP) = 13.5%) and fresh water *ad libitum.* The food supply was calculated to satisfy daily ovine requirements in late gestation (National Research Council 1985). Ten ewes each from the HA and LA groups were chosen at random to receive antioxidant vitamin supplementation, administered daily from 30 days before mating until the end of the experiment. To achieve supplementation, 0.5 kg alfalfa supplemented with 500 mg vitamin C and 350 IU vitamin E per animal was administered in individual feeding troughs early each morning. After the consumption of this ration, the remaining alfalfa was given to the ewes. As a control, an additional LA group of 10 Creole ewes kept at LA were evaluated. Half the LA ewes also received antioxidant vitamin supplementation. These animals were maintained using same management and feeding protocols as at HA. Thus, a total of six groups of pregnant ewes were studied: highland origin, HA pregnancy with (HHv) or without (HH) vitamin supplementation; lowland origin HA pregnancy with (LHv) or without (LH) vitamin supplementation; and lowland origin LA pregnancy with (LLv) or without (LL) vitamin supplementation.

Mating at HA and LA was achieved by introducing two proven males into the females' pens. Males were alternated daily between vitamin-treated and non-treated females. The chests of the rams were painted daily with a mixture of vegetable oil and coloured powder, so that any ewe in oestrous that was mounted by a male could be detected by observation of a coloured rump. Ewe bodyweight at the beginning of the mating period did not differ among the groups. The length of the mating period was 35 days. Twenty-five days after detection of mating, the ewes were screened for pregnancy by transrectal ultrasound.

Measurements in maternal blood

At 110–120 days gestation, one blood sample (5 mL) was obtained from each pregnant ewe from the left jugular vein to enable determination of plasma concentrations of vitamins C and E. In addition, these blood samples were used to determine plasma concentrations of carbonyl groups (CO) and malondialdehyde (MDA) to estimate protein and lipid oxidative damage, respectively. Total plasma antioxidant capacity (TAC)

was also assessed to complete the evaluation of oxidative stress (Griffiths *et al.* 2002).

Vitamin concentrations were measured by high-performance liquid chromatography (HPLC; Waters Alliance 2695; Waters, Milford, MA, USA). Vitamin C was measured by HPLC with amperometric detection as described by Pachla and Kissinger (1979). Briefly, plasma samples were diluted 10-fold in ultrapure water, homogenised and filtered through a 0.45-μm pore membrane. The chromatographic conditions were as follows: analytical column 250 × 4.6 mm (Symetry Shield C18, 5 μm; Waters); mobile phase (metaphosphoric acid 4 g L^{-1} ultrapure water) flow rate 1 mL min^{-1}; injection volume 10 μL; and working temperature 30°C. The detector was equipped with a glassy carbon electrode operated at 800 mV and an Ag/AgCl reference electrode. Vitamin E was measured by HPLC with fluorescence detection according to the methods of Zhao *et al.* (2004). For vitamin E extraction, plasma samples (100 μL) were diluted 1 : 1 : 6 in ethanol and dichloromethane, respectively, then centrifuged at 10 000*g* for 10 min at room temperature and the pellet was dried under a nitrogen atmosphere. The dried residue was reconstituted in methanol (200 μL). The chromatographic conditions were as follows: analytical column 150 × 4.6 mm (Symetry C18, 5 μm; Waters); mobile phase (methanol HPLC 100%) flow rate 1 mL min^{-1}; injection volume 20 μL; and working temperature 30°C. The spectrofluorimeter was set at excitation and emission wavelengths of 290 and 330 nm, respectively.

Plasma CO was determined by a spectrophotometric method, following the protocol described by Reznick and Packer (1994). Plasma MDA concentrations were measured by HPLC with fluorescence detection using the thiobarbituric acid assay described by Lästard *et al.* (2002). TAC was assessed in plasma using the total radical-trapping antioxidant parameter technique described by Wayner *et al.* (1985) and modified by Lissi *et al.* (1992).

Immediately after venous blood samples had been collected, one arterial blood sample was obtained per animal for measurement of blood gases. The arterial blood sample was drawn by puncture of the left carotid artery using a sterile, heparinised 1-mL syringe. Measurements of P_{aO_2}, P_{aCO_2}, haematocrit (Hct), haemoglobin (Hb), Hb oxygen saturation (S_{Hb}), and pH were performed immediately in an IL Synthesis 25 gas analyser (Instrumentation Laboratory, Lexington, MA, USA), calibrated to local atmospheric pressure and ovine temperature.

Newborn measurements

Immediately after birth, newborn lambs were weighed on an electronic scale (Simplex 150, Shanghai Yaohua Weighing System, Shanghai, China). In addition, biparietal diameter (BPD), abdominal diameter (AD) and thorax height (TH) were determined manually using calipers. BPD was measured as the greatest distance between the parietal bones; AD was measured transverse to the longitudinal plane, immediately rostral to the last rib; and TH was measured as the vertical distance between the sternum and the spinal column at the level of the elbows.

Placental recovery and processing

From Day 140 of pregnancy onwards, animals were observed frequently to detect external and behavioural signs of parturition. Immediately after delivery, natural expulsion of the placentas, including membranes, was awaited. Recovered placentas were rinsed with physiological buffered saline, placed in a plastic colander for 1 min to drain and weighed on an electronic scale (Excell KS-500, Manufacturas Triunfo, Santiago, Chile). Placentas were then fixed for 48 h in 10% buffered formalin before the number of cotyledons was determined. In addition, cotyledon diameter was calculated and an estimate of the surface area of cotyledon–caruncle contacts was made. Cotyledon diameter was determined by averaging the distance of the longest axis and its transverse axis at the centre of the structure. Cotyledon surface area was estimated by assuming a circumferential surface, as described by Parraguez *et al.* (2006). Placental surface area for each animal was calculated by adding up individual cotyledon–caruncle contact surface areas.

An estimate of cross-sectional placental vascular area was obtained as follows. After an additional overnight fixation period in 10% buffered formalin, three cotyledons per animal were processed for light microscopic histology. Serial 6-μm sections from the middle zone through the transverse plane were obtained and stained with haematoxylin–eosin. Measurement of the cotyledon area occupied by the vasculature was determined in four sections and four microscopic fields of 151 386 μm^2 per section using an Eclipse E-600 microscope (Nikon, Tokyo, Japan). Images were captured with ×100 augmentation using a digital video camera (Cool Snap-Pro; Media Cybernetics, Bethesda, MD, USA) coupled to the microscope. In each image, cross-sectional cotyledon area occupied by the vasculature as a fraction of the cotyledon tissue area was calculated using Image Pro-Plus morphometric analysis software (Media Cybernetics). This value was then multiplied by the placental surface area for each animal, obtaining a value for cross-sectional placental vascular area per animal. These values were averaged to obtain a cross-sectional placental vascular area per group. In this analysis, medium to large vessels were mainly considered because most of the microvessels had collapsed or were destroyed at the time of delivery.

Statistical analyses

Data were compared by analysis of variance using the general linear model (GLM) procedure in SAS (SAS, Cary, NC, USA). Comparisons were made using two statistical models. The first model was used for gestations at HA and consisted of a factorial model with two factors: origin (place where the ewes that gave birth were born) and the vitamin supplementation (presence or absence). The second model took into consideration the previous two factors in addition to the place in which gestations took place. Interactions among factors were also analysed. When significant differences were found, Duncan's test was used to determine significant differences among groups. $P \leq 0.05$ was considered significant. Results are expressed as the mean ± s.e.m.

Results

No effect of sire was found on maternal or newborn lamb variables.

Maternal variables

All pregnancies resulted in normal parturition and healthy newborns. Maternal bodyweight on Day 140.2 ± 0.7 of gestation in the HH, HHv, LH, LHv, LL and LLv groups was 46.7 ± 1.1, 47.5 ± 1.7, 47.2 ± 1.3, 46.4 ± 1.5, 48.3 ± 1.1 and 47.9 ± 1.5 kg, respectively. There were no significant differences among the six groups.

Plasma concentrations of vitamins C and E are shown in Fig. 1*a*, *b*. Antioxidant vitamin supplementation significantly increased plasma concentrations of both vitamins C and E. Plasma vitamin C levels in treated ewes were approximately twofold greater than levels in untreated ewes ($P < 0.05$). No effect of origin or gestation altitude on plasma vitamin C levels was found, although a significant interaction between origin and vitamin treatment was observed (i.e. levels in the HH group were higher than those in the LH group; $P < 0.03$). No interaction was observed when the altitude at which the pregnancy took place was included in the statistical model. This is due to the fact plasma vitamin C levels in the LL group were the same as in the HH and LH groups. Compared with levels in untreated ewes at the same altitude, plasma vitamin E levels were twofold greater in the vitamin-supplemented HA gestation groups and threefold greater in the vitamin-supplemented LA gestation group ($P < 0.05$). These differences appeared to be due to interactions

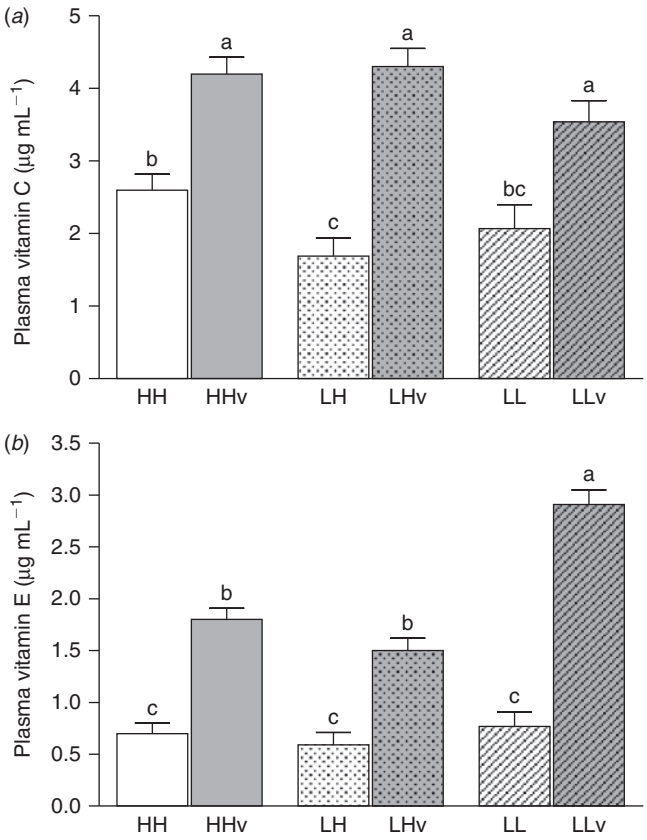

Fig. 1. Plasma concentrations of vitamins C (*a*) and E (*b*) in pregnant ewes at high altitude either supplemented daily with vitamins C and E (HHv, high altitude natives; LHv, low altitude natives) or not (HH, high altitude natives; LH, low altitude natives). LLv and LL, control pregnancies at low altitude with and without vitamin supplementation, respectively. Columns with different letters differ significantly ($P < 0.05$, Duncan's test). Data are presented as mean ± s.e.m.

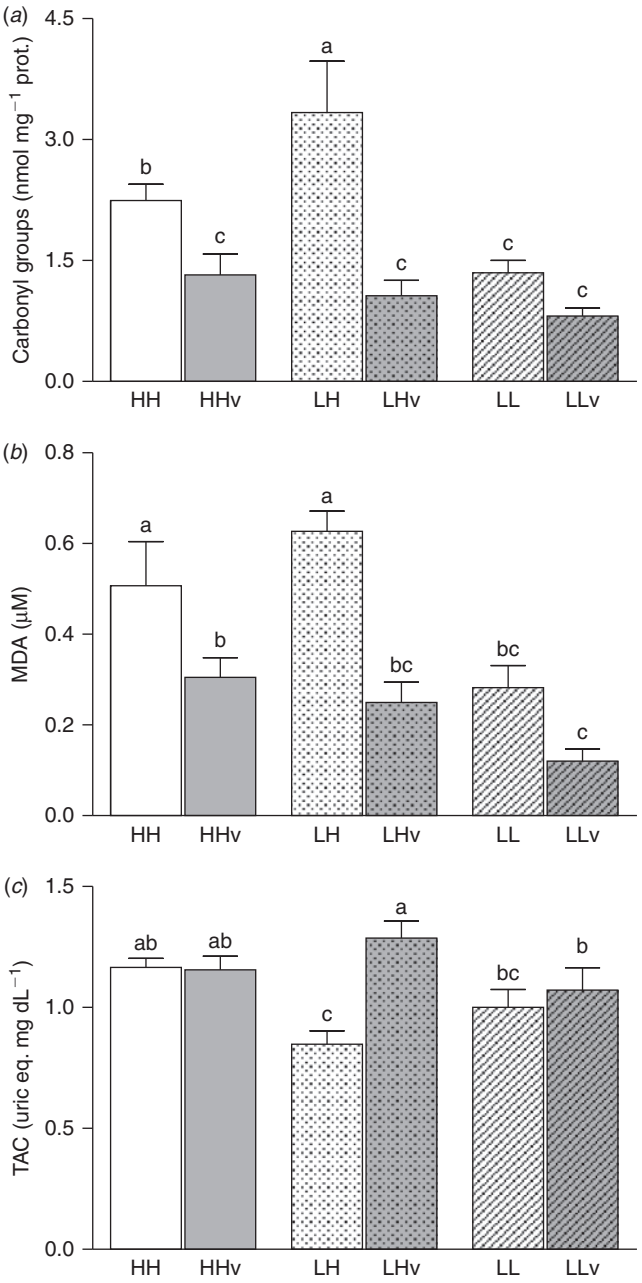

Fig. 2. Plasma concentrations of carbonyl groups (*a*) and malondialdehyde (*b*), as well as total antioxidant capacity (*c*) in pregnant ewes at high altitude either supplemented daily with vitamins C and E (HHv, high altitude natives; LHv, low altitude natives) or not (HH, high altitude natives; LH, low altitude natives). LLv and LL, control pregnancies at low altitude with and without vitamin supplementation, respectively. Columns with different letters differ significantly ($P < 0.05$, Duncan's test). Data are presented as mean ± s.e.m.

between origin, altitude of gestation and vitamin E administration ($P < 0.01$).

In pregnancies developed at LA, no significant effect of vitamin administration was observed on variables of oxidative status (Fig. 2) or on blood gases (Table 1).

Maternal plasma concentrations of biomarkers of oxidative damage are shown in Fig. 2*a*, *b*. Plasma CO and MDA levels were higher in HA pregnancies ($P < 0.05$). However, vitamin supplementation significantly attenuated the increases in biomarkers of oxidative damage, with values in vitamin-treated HA ewes similar to those seen in both vitamin-treated and untreated LA ewes. TAC levels (measured as mg mL^{-1} uric acid equivalents) were the lowest in the LH and LL groups ($P < 0.05$). Vitamin administration increased TAC levels in LHv ewes ($P < 0.05$; Fig. 2*c*). No differences were detected when comparing origin and altitude of pregnancy. Plasma CO and TAC levels showed significant interactions between origin and vitamin administration (for plasma CO, levels in the LH group were higher than those in the HH group, while for TAC, levels in the HH were higher in those in the LH group; $P < 0.01$), as well as between these factors and the altitude where gestation took place ($P < 0.004$).

Arterial blood gas measurements obtained near term are given in Table 1. A hypoxaemic state was observed in all HA groups. However, $P_{a}O_2$ levels were higher in both HA vitamin-treated groups ($P < 0.05$). $P_{a}CO_2$ levels were lower in ewes gestating at HA ($P < 0.05$) and there were no significant differences among the HA three groups; that is, vitamin supplementation had no effect on plasma $P_{a}CO_2$ levels. Both Hct and Hb concentrations were higher in pregnancies at HA ($P < 0.05$). Vitamin supplementation tended to increase Hct in the ewes, although this difference did not reach statistical significance. Hb concentrations were affected by an interaction between origin and vitamin administration ($P < 0.05$), whereas Hct concentrations were affected by an interaction between these factors and the altitude at which the pregnancy took place ($P < 0.05$). Values of S_{Hb} were lower in the HA groups, with values in the LH ewes being the lowest ($P < 0.05$). There were no significant differences in pH between the six groups.

Pregnancy outcomes

Pregnancy characteristics and outcomes are listed in Table 2. Vitamin supplementation had no significant effect on pregnancies at LA. The length of gestation was longer in native HA

Table 1. Blood gases in high altitude native and newcomer ewes gestating at high altitude (110–120 days gestation) and either supplemented or not with vitamins C and E

Data are the mean ± s.e.m. Different superscript letters within columns indicate significant differences between groups ($P < 0.05$, Duncan's test). Hct, hematocrit; Hb, haemoglobin concentration; S_{Hb}, haemoglobin oxygen saturation; HH, high altitude (HA) native ewes that gestated at HA without vitamin supplementation; HHv, HA native ewes that gestated at HA with vitamin supplementation; LH, low altitude (LA) native ewes that gestated at HA without vitamin supplementation; LHv, LA native ewes that gestated at HA with vitamin supplementation; LL, LA native ewes that gestated at LA without vitamin supplementation; LLv, LA native ewes that gestated at LA with vitamin supplementation

Group	$P_{a}O_2$ (mmHg)	$P_{a}CO_2$ (mmHg)	Hct (%)	Hb (mg dL^{-1})	S_{Hb} (%)	pH
HH	53.0 ± 1.2^{c}	23.8 ± 0.9^{b}	32.2 ± 0.7^{bc}	12.5 ± 0.5^{b}	82.6 ± 2.8^{b}	7.510
HHv	60.4 ± 1.3^{b}	25.8 ± 0.9^{b}	33.3 ± 0.7^{ab}	12.9 ± 1.1^{b}	85.9 ± 3.9^{b}	7.458
LH	49.5 ± 1.4^{c}	25.0 ± 1.0^{b}	35.0 ± 0.7^{a}	14.3 ± 0.4^{a}	77.5 ± 3.4^{c}	7.497
LHv	58.8 ± 1.4^{b}	25.4 ± 1.0^{b}	33.2 ± 0.8^{ab}	13.4 ± 1.4^{ab}	85.5 ± 3.2^{b}	7.474
LL	95.9 ± 1.6^{a}	39.2 ± 1.1^{a}	28.3 ± 0.8^{d}	10.5 ± 0.6^{c}	96.8 ± 2.0^{a}	7.482
LLv	98.3 ± 1.6^{a}	40.3 ± 1.2^{a}	30.5 ± 0.9^{cd}	10.9 ± 0.7^{c}	98.2 ± 2.2^{a}	7.495

Table 2. Effects of vitamins C and E on pregnancy outcomes in high and low altitude native ewes gestating at high altitude

Data are the mean ± s.e.m. Different superscript letters within columns indicate significant differences between groups ($P < 0.05$, Duncan's test). BPD, biparietal diameter; TH, thorax height; AD, abdominal diameter; HH, high altitude (HA) native ewes that gestated at HA without vitamin supplementation; HHv, HA native ewes that gestated at HA with vitamin supplementation; LH, low altitude (LA) native ewes that gestated at HA without vitamin supplementation; LHv, LA native ewes that gestated at HA with vitamin supplementation; LL, LA native ewes that gestated at LA without vitamin supplementation; LLv, LA native ewes that gestated at LA with vitamin supplementation

Group	Length of gestation (days)	Newborn weight (kg)	Newborn BPD (cm)	Newborn TH (cm)	Newborn AD (cm)
HH	152.1 ± 4.1^{a}	3.26 ± 0.15^{c}	6.38 ± 0.14^{bc}	10.87 ± 0.49	9.08 ± 1.68^{ab}
HHv	151.9 ± 4.2^{a}	3.83 ± 0.13^{b}	7.02 ± 0.13^{a}	11.29 ± 0.15	9.26 ± 2.32^{ab}
LH	145.0 ± 7.6^{b}	2.98 ± 0.14^{c}	6.29 ± 0.15^{c}	11.23 ± 0.53	8.74 ± 1.60^{b}
LHv	150.6 ± 4.5^{ab}	3.60 ± 0.15^{b}	6.66 ± 0.15^{abc}	11.00 ± 0.14	8.69 ± 1.11^{b}
LL	145.4 ± 2.5^{b}	4.38 ± 0.20^{a}	6.86 ± 0.20^{ab}	10.50 ± 0.24	10.82 ± 0.71^{a}
LLv	146.2 ± 3.8^{b}	4.26 ± 0.20^{a}	7.04 ± 0.17^{a}	10.66 ± 0.28	10.50 ± 1.03^{ab}

Table 3. Effects of vitamins C and E on placental characteristics in high and low altitude native ewes gestating at high altitude

Data are the mean ± s.e.m. Different superscript letters within columns indicate significant differences between groups ($P < 0.05$, Duncan's test). HH, high altitude (HA) native ewes that gestated at HA without vitamin supplementation; HHv, HA native ewes that gestated at HA with vitamin supplementation; LH, low altitude (LA) native ewes that gestated at HA without vitamin supplementation; LHv, LA native ewes that gestated at HA with vitamin supplementation; LL, LA native ewes that gestated at LA without vitamin supplementation; LLv, LA native ewes that gestated at LA with vitamin supplementation

Group	Placental weight (g)	No. cotyledons	Cotyledon diameter (cm)
HH	380.9 ± 16.7^{a}	55.2 ± 4.5^{b}	2.4 ± 0.5^{a}
HHv	305.2 ± 15.9^{b}	83.4 ± 4.3^{a}	1.4 ± 0.3^{b}
LH	285.5 ± 18.7^{b}	56.6 ± 5.1^{b}	1.9 ± 0.3^{ab}
LHv	313.0 ± 17.6^{b}	78.6 ± 4.5^{a}	1.3 ± 0.2^{b}
LL	308.5 ± 23.6^{b}	82.0 ± 6.1^{a}	1.5 ± 0.3^{b}
LLv	323.4 ± 24.6^{b}	79.4 ± 9.8^{a}	1.4 ± 0.3^{b}

ewes than in native LA ewes ($P < 0.05$), whereas gestational length was intermediate in the LHv group. Neither vitamin supplementation nor the altitude at which gestation took place affected gestation length. The weight of newborn lambs was significantly lower in HA pregnancies ($P < 0.05$), with the lowest values recorded for lambs born to LH ewes. The weight of newborn lambs was increased significantly following vitamin supplementation of HA groups compared with the untreated HA groups ($P < 0.05$). The greatest difference in the weight of newborn lambs was between the LH and LHv groups. The BPD of newborn lambs was shorter for the HA groups ($P < 0.05$). Vitamin supplementation increased BPD to values observed at in lambs born at LA. There were no significant differences in TH between lambs in the six groups. The AD of newborn lambs was only affected by the altitude at which gestation took place, with AD being lowest in the LH and LHv groups ($P < 0.05$).

Placental characteristics

Vitamin supplementation had no effect on any placental characteristics in pregnancies developed at LA (Table 3; Fig. 3).

Placental weight, the number of cotyledons and cotyledon diameter are listed in Table 3. Placental weight was greater in HA native ewes ($P < 0.05$). Vitamin supplementation decreased placental weight in this group, resulting in placental weight that was similar to that seen in LA native ewes. Interactions for this trait were observed between origin and vitamin administration ($P < 0.004$), as well as between these factors and gestational altitude ($P < 0.008$). The number of cotyledons was decreased significantly in pregnancies at HA ($P < 0.05$). Vitamin supplementation increased the number of cotyledons in these groups to match values obtained in ewes at LA. An interaction ($P < 0.02$) for this trait was found when origin, gestational altitude and vitamin effects were included in the statistical model. Cotyledon diameter was highest in HH ewes ($P < 0.05$), followed by LH ewes. Vitamin supplementation significantly decreased cotyledon diameter in HA ewes ($P < 0.05$) to values similar to those in LA pregnancies.

Placental surface area and cross-sectional placental vascular area are shown in Fig. 3*a*, *b*. The placental surface area was increased by both HA origin and HA gestation ($P < 0.05$). Thus, the HH group had the greatest placental surface area, followed by the LH group. Vitamin supplementation decreased the placental surface area in both groups of ewes at HA ($P < 0.05$). Significant interactions were found between origin and vitamin administration ($P < 0.001$), as well as between these factors and the altitude at which gestation took place ($P < 0.001$). Cross-sectional placental vascular area was increased in HA native

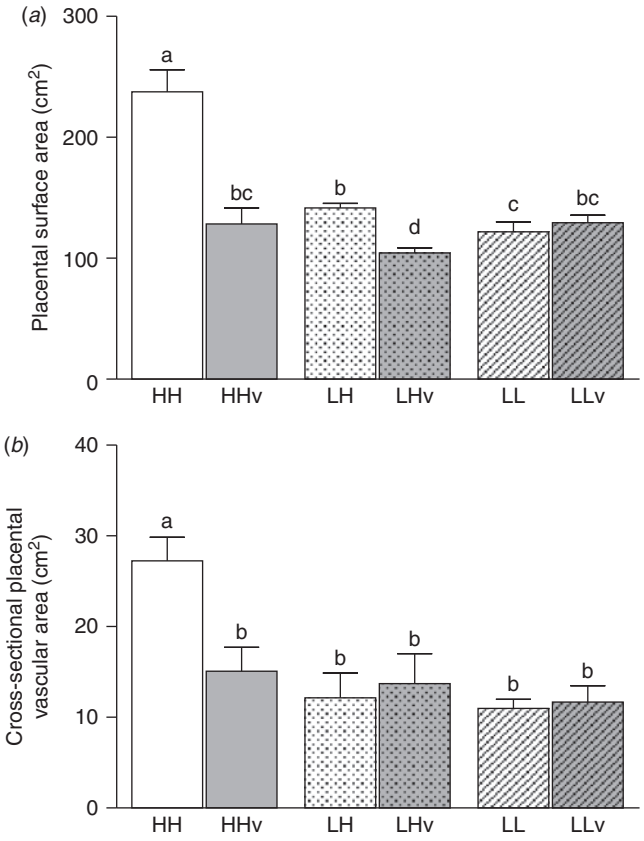

Fig. 3. Placental surface area (*a*) and cross-sectional placental vascular area (*b*) in pregnant ewes at high altitude either supplemented daily with vitamins C and E (HHv, high altitude natives; LHv, low altitude natives) or not (HH, high altitude natives; LH, low altitude natives). LLv and LL, control pregnancies at low altitude with and without vitamin supplementation, respectively. Columns with different letters differ significantly ($P < 0.05$, Duncan's test). Data are presented as mean ± s.e.m.

ewes ($P < 0.05$), whereas vitamin treatment decreased this trait in ewes from the same origin ($P < 0.05$). Neither the altitude at which gestation took place nor vitamin supplementation had any effect in LA ewes. Significant interactions between origin and vitamin treatment were found for placental vascularity ($P < 0.01$), as well as between these factors and the gestational altitude ($P < 0.009$).

Discussion

To our knowledge, this is the first study to characterise redox homeostasis and the effect of antioxidant vitamin supplementation on sheep pregnancy at HA. The present study demonstrates a significant effect of antioxidants on preventing oxidative stress and its undesirable consequences on fetal birthweight and placental characteristics in hypoxic pregnancies, thereby abolishing most of the differences between pregnancies in animals that have resided at HA for a long or short time.

Maternal blood variables

It is commonly accepted that ruminants synthesise their own vitamin C to satisfy dietary needs. However, under certain stressful environmental conditions, such as at cold temperatures, exogenous supplementation is necessary to avoid the effects of vitamin C deficiency (Black and Hidiroglou 1996). The success of oral administration of vitamin C in increasing serum concentrations in ruminants is contentious. It has been reported that blood levels of vitamin C did not increase in ruminants following oral administration (Knight *et al.* 1941). However, more recent work has demonstrated significant increases in vitamin C plasma concentrations after administration in cows (Hidiroglou 1999). The results of the present study are in agreement with this later work, indicating that daily oral administration of vitamin C to pregnant ewes over a longer period (~150 days) significantly increases the plasma levels of this vitamin. In the case of vitamin E, the increase observed in plasma levels in supplemented ewes in the present study was generally consistent with that reported previously (Ochoa *et al.* 1992; Njeru *et al.* 1994; Capper *et al.* 2005). The increases in plasma vitamin concentrations in supplemented pregnant ewes are of considerable importance in terms of vitamin transfer to the fetus. In fact, the results of fetal lamb growth and newborn outcomes, as reported in the present study, are strongly supported by previous demonstration of a direct relationship between maternal and newborn plasma levels of vitamin E (Capper *et al.* 2005), reflecting trans-placental transfer of this vitamin. Furthermore, evidence of early trans-placental transfer of vitamins C and E has been reported in human pregnancies (Jauniaux *et al.* 2004). Taking into account the anatomical and developmental differences between human and sheep placentas, this information supports our results in newborn lambs.

A state of oxidative stress was observed in pregnancies at HA, as demonstrated by the significant increase in plasma concentrations of CO and MDA. This state was accentuated in ewes new to the HA environment relative to native HA ewes. The difference in the magnitude of the hypoxia-induced oxidative stress between the two groups may be due to an adaptive mechanism present in native HA ewes. A similar finding was reported recently in native highlander and sojourner humans after long exposure to HA (Sinha *et al.* 2009). Despite the CO and MDA results in the present study, no concordant changes in TAC levels were observed. This apparent inconsistency has been described in other experiments at HA. For example, it has been demonstrated in humans that while working in a hypobaric hypoxic environment, changes in oxidative stress biomarkers are not necessarily associated with changes in TAC levels (Chao *et al.* 1999), probably due to variations in the timing of the expression of the different biomarkers in plasma (Vij *et al.* 2005). Furthermore, it is of note that supplementation with vitamins C and E in the present study significantly lowered levels of biomarkers of oxidative stress in HA pregnancies to values observed in pregnancies at LA, and significantly increased TAC levels in the plasma of ewes that were newcomers to the HA environment. These results demonstrate that antioxidant therapy in gestational ewes at HA is effective in preventing oxidative stress.

Despite the effects described above, an evident hypoxaemic state was present in the ewes at HA, and values for all blood parameters were similar to those reported previously (Parraguez *et al.* 2006). The administration of antioxidant vitamins had an interesting effect on the blood features involved in oxygen transport in HA animals. Significant increases in $P_{a}O_2$ (13.9–18.8%) and in S_{Hb} (4.4–10.3%) were observed, suggesting a protective effect of the vitamins on pulmonary function under HA hypoxia. It has been demonstrated that acute or chronic exposure to hypoxia, as well as oxidative stress, induce lung injury and diminish gas exchange (Tuder *et al.* 2007; Park *et al.* 2009). It has also been demonstrated that the administration of antioxidants in rats exposed to hypobaric hypoxia prevents hypoxia-induced pulmonary damage and results in significantly decreased MDA levels in the blood as well as an increase in total antioxidant status (Uzun *et al.* 2006). Although we did not examine lung structure and function as part of the present study, further work may explain the improvement in blood oxygen transport features observed in our study; there may be a preventative effect of the vitamins on hypoxia/oxidative stress-induced pulmonary damage. The observed differences in blood oxygen pressure and Hb saturation in response to antioxidant vitamin therapy may provide better oxygenation to the fetal–placental unit and thus be one of the factors responsible for birthweight recovery. This effect would be increased by the rightward shift in the Hb affinity curve, as has been demonstrated under hypobaric hypoxic conditions (West 2002).

Pregnancy outcome

In a previous study of pregnant sheep, we reported the effects of natural HA hypobaric hypoxia on newborn outcomes (Parraguez *et al.* 2005), which were the result of slow fetal growth resulting in low birthweight. The present study confirms our previous findings and demonstrates that vitamins C and E, supplied daily during pregnancies at HA, diminished the effect of hypobaric hypoxia on newborn birthweight and BPD, resulting in values that were comparable to those observed at LA (Parraguez *et al.* 2005). From our point of view, one of the most

impressive effects of the antioxidant vitamin supplementation in pregnant ewes at HA, independent of the time of exposure to this environment, was the significant increase (~20%) in lamb bodyweight at birth. This increase was a specific effect of the vitamin treatment at HA, because vitamin supplementation at LA had no effect. Considering the improvement in the mortality, morbidity (Nash *et al.* 1996) and growth rates of the lambs as a function of birthweight (Villete and Theriez 1981), this vitamin supplementation may have a significant beneficial effect on the health and production of HA sheep livestock, with subsequent economic benefits for HA sheep breeders. There is no information on the effect of antioxidant therapy on fetal growth in pregnancies under hypobaric hypoxia. However, different types of experiments have demonstrated the beneficial effects of antioxidant therapy on several physiological responses to HA or hypoxia (Inan *et al.* 1995; Kelly and Richardson 1996; Tan *et al.* 1996; Mohanraj *et al.* 1998; Purkayastha *et al.* 1999; Bailey and Davies 2001; Ilavazhagan *et al.* 2001; Miura *et al.* 2006). In addition, several studies have shown that, in pathological gestations in which oxidative stress is evident, as in pre-eclampsia (Chamy *et al.* 2006) or diabetic pregnancies (Cederberg *et al.* 2001*a*), vitamins C and E are successful in preventing oxidative fetal damage and improving fetal outcome (Cederberg *et al.* 2001*b*; Chappell *et al.* 2002; Rodrigo *et al.* 2005). However, recent large multicentre clinical studies have not been able to confirm the benefits of vitamins C and E in pregnancies at risk of pre-eclampsia (Poston *et al.* 2006; Villar *et al.* 2009; Xu *et al.* 2010). Moreover, an increased rate of low birthweight babies (Poston *et al.* 2006) and increased risk of fetal loss, perinatal death or preterm labour has been reported (Xu *et al.* 2010). However, there are some methodological drawbacks in these latter studies (i.e. populations with different risks of pre-eclampsia, coexisting illness, unknown status of plasma vitamin concentrations, blood concentrations achieved with vitamin supplementation not determined, no measurements of biomarkers of oxidative stress in the plasma, etc.), limiting the interpretation of their results. Conversely, there is a complementary body of evidence that lends support to the role of antioxidant status on fetal outcome. Thus, one study in women with low birthweight babies detected high concentrations of oxidative stress biomarkers in the blood showed that diminished after vitamin C treatment (Karowicz-Bilińska *et al.* 2002). It is important to note that in human pregnancies at term there is a positive correlation between maternal and fetal biomarkers of oxidative stress (Argüelles *et al.* 2006). Furthermore, in other studies performed in healthy pregnant women, a direct relationship between plasma concentrations of vitamins C and E at 24–28 weeks gestation and birthweight was seen (Lee *et al.* 2004; Min *et al.* 2006), in addition to an inverse relationship between plasma vitamin C concentrations at 37 weeks gestation and trophoblast apoptotic activity (Ahn *et al.* 2007). These relationships suggest a key role for antioxidant status on maternal oxidative stress, placental integrity and fetal growth.

Placental changes

Ovine placental tissue is very susceptible to environmental stressors, including HA hypoxia (Parraguez *et al.* 2006), poor nutrition (Vonnahme *et al.* 2003) and high temperature (Galan *et al.* 1999). In a previous study, we demonstrated several placental changes (increased placental weight, cotyledon diameter, placental surface area and cotyledon area occupied by the vascular lumina, as well as a decreased number of cotyledons per placenta) as a compensatory response to maternal–fetal hypoxic stress. Moreover we demonstrated that, in our experimental model, the effects of poor nutrition and high environmental temperatures on these parameters were negligible (Parraguez *et al.* 2006). Recently, we reported that these placental changes were associated with the placental overexpression of the pro-angiogenic factors vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS; Parraguez *et al.* 2010). In the present study, we observed that, in general, supplementation with vitamins C and E had no effect on LA pregnancies, but it prevented the effects of HA hypoxia on most of the placental characteristics described above in HA-adapted ewes. A less significant effect of vitamin supplementation was seen in HA newcomer ewes, in which only a decrease in the number of placentomes and placental area, as well as tendency for decreased cotyledon diameter, were evident. Indeed, in both groups supplemented with vitamins at HA, there was a trend for the placental parameters to move towards those seen in ewes at LA (Parraguez *et al.* 2006). During pregnancy, the development and function of the placenta depend primarily on the growth of its vascular bed, which is controlled by several angiogenic factors (Reynolds *et al.* 2005). Thus, the main effects of vitamins on hypoxic placentas may be explained by a reduction in the local expression of angiogenic factors, resulting in a reduction in placental vessels and mass. As shown in the present study, antioxidant vitamin supplementation increases oxygen transport and reduces oxidative stress, compensating for the effect of the hypoxic environment. Under these conditions, hypoxia inducible factor (HIF)-1, the protein responsible for the activation of several angiogenic factors, including VEGF (Carmeliet *et al.* 1988), may be diminished as a result of a reduced expression of the HIF-1α subunit, thus decreasing expression of the angiogenic factors. As a result of decreased VEGF (Bouloumié *et al.* 1999) or as a direct effect of ameliorated oxygen tension (Sladek *et al.* 1997), eNOS may be also reduced. There is no information available on the effect of antioxidant vitamins on placental angiogenesis and its regulating factors. In a chicken model of angiogenesis, inhibition of angiogenesis by antioxidants has been reported (Polytarchou and Papadimitriou 2004), which may support our hypothesis. However, this should be confirmed with future experiments.

Comparing the results of vitamin supplementation on the weight of newborns and placental changes, an apparent contradiction emerges. If we consider that placental surface area and its cross-sectional vascular area should be directly associated with the maternal–fetal transfer of oxygen and other nutrients, we may expect that the improvement of newborn weight, as observed in ewes receiving antioxidant vitamin supplementation in the present study, would be the result of an increase in these placental traits. However, in the present study, antioxidant vitamin supplementation resulted in the opposite placental response. Even though the method used to measure vascularity in the present study did not enable us to determine placentome

microvascularity, and so the functional exchange area, changes detected in the medium and large vessels could reflect the existence of stimuli for variations in total vascular growth. Our interpretation of these findings is that, despite the reduction in placental surface area and cross-sectional vascular area, the antioxidant vitamin treatment induced some mechanism that increased overall placental vascular blood flow. There is no published information on the effect of antioxidant vitamins on ovine placentas in normal or pathological pregnancies. However, the beneficial effect of antioxidant vitamin therapy in human pregnancies complicated by hypertension (e.g. as a result of pre-eclampsia or diabetes) may be associated with recovery of vascular responses to vasodilator agents, which were previously diminished by oxidative stress (Kossenjans *et al.* 2000).

Several placental changes have been reported in human pregnancies at HA (Zhang *et al.* 2002; Tissot van Patot *et al.* 2003, 2004; Zamudio 2003) that are consistent with those observed in ewes at HA (Parraguez *et al.* 2006). We have not found any study in which antioxidants were administered to pregnant women at HA to prevent oxidative stress. However, in pre-eclampsia, a pathological condition characterised by placental hypoperfusion, increased vascular resistance, proteinuria and, frequently, low birthweight babies (Hubel 1999), it has been postulated that oxidative stress is the fundamental abnormality leading to endothelial damage and the presentation of clinical symptoms (Noris *et al.* 2005). Furthermore, a greatly elevated risk of pre-eclampsia in pregnant women at HA has been reported (Keyes *et al.* 2003). Most of the evidence in these studies indicates a close association between low plasma antioxidant status, especially plasma vitamin C concentrations, and development of the syndrome (Hubel 1999; Chappell *et al.* 2002; Rodrigo *et al.* 2005; Chamy *et al.* 2006; Mehendale *et al.* 2008; Karacay *et al.* 2010). In addition, decreased concentrations of vitamin C in umbilical venous plasma and in placental tissue homogenates in pre-eclamptic women has been reported (Kim *et al.* 2006) and a beneficial role of vitamins C and E in preventing pre-eclampsia has been demonstrated (Chappell *et al.* 1999, 2002; Rodrigo *et al.* 2005; Rumiris *et al.* 2006). The effects of antioxidant therapy in pre-eclampsia are associated with decreased endothelial damage, amelioration of placental blood flow and an improvement of other symptoms (Noris *et al.* 2005). This information supports our interpretation of the data and constitutes a strong basis on which to consider the effectiveness of supplementation with vitamins C and E in preventing the effects of HA oxidative stress on the placental–fetal environment.

Conclusion

The present results allow us to conclude that supplementation with vitamins C and E during ovine gestation at HA prevents maternal hypoxia-induced oxidative stress and its effects on placental characteristics. Moreover, both HA and LA native pregnant ewes respond satisfactorily to the effect of these antioxidant vitamins, with increased newborn bodyweight and an abolition of the differences observed between these groups of animals in the absence of daily antioxidant administration. Even though we acknowledge that the absence of a highland origin–lowland pregnancy group is a probable limitation of the present study, the results allow us to highlight the possibility of using supplementation with vitamins C and E as an economic tool to improve ovine production at HA.

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