Redox, acid-base and clinical analysis of preterm and term neonatal lambs

Lieve Cristina Garcia Silva, Fernanda Machado Regazzi, Cristina Fátima Lúcio, Gisele Almeida Lima Veiga, Daniel Souza Ramos Angrimani, Claudia Barbosa Fernandes, Camila Infantesi Vannucchi

Department of Animal Reproduction, School of Veterinary Medicine and Animal Science, University of São Paulo, São Paulo, SP, 05508-270, Brazil.

Abstract

During pregnancy, fetal lambs are exposed to low oxygen tension. Thus, an effective antioxidant mechanism is partially developed which sensitizes fetus to oxidative stress. Consequently, term and preterm neonates are susceptible to molecular and cellular injury caused by oxygen species (ROS). This study aimed to evaluate the development of antioxidant enzymes and oxidative profile of preterm (135 days of pregnancy) and term (145 days of pregnancy) neonatal lambs, correlating with clinical analysis. Preterm lambs had significantly (P ≤ 0.05) lower score of vitality (4.00 ± 1.10), bradycardia (99 ± 34 bpm) and bradypnea (13 ± 10 mpm). However, both groups were normothermic and eucloric. Preterm group had low blood pH (7.07 ± 0.10) and both groups had hypercapnia, more severe in preterm group (85.52 ± 1.16 mmHg). In addition, preterm newborns had lower pO2 (10.67 ± 5.65 mmHg) and SO2 (6.17 ± 5.85%) values. No significant difference (P ≥ 0.05) was verified on antioxidant enzymes and oxidative stress were among experimental groups, although glutathione peroxidase negatively correlated with Apgar score, heart rate, SO2 and pO2. Our data show that preterm neonates are less adapted to the odds of labor and to overcome the immediate changes of extra-uterine life. Furthermore, we verified an influence of glutathione peroxidase in controlling oxidative stress, which highlights mature enzymatic mechanisms of cell redox, even in premature lambs.

Keywords: glutathione peroxidase, lung, neonate, superoxide dismutase, TBARS.

Introduction

The neonatal period represents an adaptive phase in which clinical outcome is inwardly related to physiological maturity. Neonatal mortality rate during the first weeks in lambs is estimated to be higher than to 15% (Dwyer and Morgan, 2006). However, in premature lambs, such percentage can be much higher, resulting in significant economic losses to the sheep industry.

During pregnancy, fetal lambs are exposed to low oxygen tension (O2; Gittto et al., 2002). Consequently, the system of combating free radicals is partially developed and the imbalance between pro- and anti-oxidant systems sensitizes fetus to oxidative stress. With the onset of aerobic metabolism after birth, there is a significant increase in O2 consumption, which triggers the production of free radicals in the mitochondrial respiratory chain (Vlessis and Mela-Riker, 1989). Of the total O2 consumed by cells, approximately 5% are metabolized into reactive oxygen species (ROS) such as superoxide anion (O2·), hydrogen peroxide (H2O2) and hydroxyl radical (OH). Thus, the excessive production of ROS without a specific recombination complex is responsible for molecular and cellular injury to different body tissues, especially the brain and lung (Gittto et al., 2002). The mechanism of oxygen toxicity is attributed to the direct or indirect action of free radicals in cellular components, leading to, for example, enzyme denaturation, disturbances in intracellular calcium homeostasis and cell death (Freeman and Crapo, 1982; Rodrigues, 1998).

In premature human newborns, oxidative stress is even more severe than for term births (Perrone et al., 2010). The premature infant is especially susceptible to ROS damage because adequate concentrations of antioxidants are absent at birth, due to low maternal-fetal placental transfer and endogenous production; and the ability to increase the antioxidant synthesis in response to hyperoxia is deficient (Davis and Auten, 2010). Hypoxic preterm neonates present high levels of plasma free radicals and protein oxidation metabolites at delivery and at seven days of life (Buonocore et al., 2002).

The injuries caused by O2 free radicals are counteracted by enzymatic and non-enzymatic antioxidant defenses, such as superoxide dismutase (SOD), catalase and glutathione peroxidase (GPx) and non-enzymatic antioxidant defenses, for example, vitamins E, C, uric acid and bilirubin. Antioxidant profile of human newborns is marked by low levels of GPx, SOD, beta-carotene, riboflavin, riboproteinase, vitamin E, selenium, among other factors (Gopinathan et al., 1994). In veterinary medicine, studies on antioxidant competence in the newborn sheep are yet to be performed.

In human fetus, there is a parallel of the antioxidant maturation pattern with the pulmonary surfactant system, with a 150% increase in SOD, GPx and catalase during the last 15% of gestation (Davis and Auten, 2010). Therefore, we hypothesize that the redox system is related to acid-base balance after birth and premature lambs have both an antioxidant and clinical impairment compared to term neonates, which leads to the necessity of a specific reanimation protocol. Thus, the aims of this study were to evaluate the development of the antioxidant system at the end of gestation and the oxidative profile of preterm and term neonatal lambs, correlating with the clinical analysis immediately after birth.

Materials and Methods

The present study complied with the ethical requirements for the use of animals in experiments, and
was approved by the Bioethics Committee of the Faculty of Veterinary Medicine and Animal Science, University of São Paulo (protocol number 2039/2010).

Animals and experimental design

A total of 12 newborn lambs of both genders were analyzed, divided into 2 groups according to gestational age at birth: preterm group (135 days of pregnancy; n = 6), term group (145 days of pregnancy; n = 6).

We used eight clinically healthy Santa Ines ewes, aged from 2 to 5 years, raised in an intensive system. Females were fed a commercial balanced ration, hay and mineral supplementation twice daily and water was provided ad libitum. Ewes were subjected to heat induction protocol with a single injection of 2.5 mg tromethamine dinoprost (Lutalyse®, Pfizer) via IM. Clinical, acid-base and glucose neonatal analysis

The measurement of thiobarbituric acid reactive substances (TBARS), aiming to access malondiadehyde levels, was made in accordance with a protocol first described by Ohkawa et al. (1979). To precipitate proteins, 200 µl of serum and 400 µl of a 10% solution (v:v) of trichloroacetic acid (TCA 10%) were mixed and centrifuged (18,000 g for 15 min at 15°C). After centrifugation, 500 µl of the supernatant and 500 µl of 1% (v:v) thiobarbituric acid (TBA, 1%), in 0.05N sodium hydroxide in glass tubes were placed into a boiling water bath (100°C) for 10 min, and subsequently cooled in an ice bath (0°C) to stop the chemical reaction. TBARS were quantified by spectrophotometry, with a wavelength of 532 nm (Ultrspec 3300 Pro®, Amersham Biosciences). The results were compared to a standard curve previously made with a solution of malondiadehyde (MDA), using the value of 1.56 × 105 M−1 cm−1 as the MDA extinction coefficient (Buege and Aust, 1978). The lipid-peroxidation index was described as nanograms of TBARS/ml of serum.

The GPx activity was measured by spectrophotometry (Ultrspec 3300 Pro®, Amersham Biosciences) in a wavelength of 340 nm at 37°C for 100 min (Nichi et al., 2007), followed by a reaction containing nicotinamide adenine dinucleotide phosphate (NADPH; 0.12 mM, 1 ml), oxidized glutathione (GSSGr; 0.25 U/ml, 20 ml) and GSH (1 mM, 100 ml). The results were expressed in U/ml.

The determination of serum SOD activity was performed according Flohe and Otting (1984). The assay was performed in a spectrophotometer (Ultrspec 3300 Pro®, Amersham Biosciences) at 550 nm and 25°C in a reaction medium containing cytochrome C (1 µm), xanthine (50 M), EDTA (100 µm) and sodium phosphate buffer (50 µm, pH 7.8). The results were expressed as U/ml.

Statistical analysis

The results values were compared using SAS for Windows (SAS Institute Inc., Cary, NC, USA,
2000). The effect of gestational age groups (preterm vs. term) was determined using parametric (T test) and nonparametric (Wilcoxon) tests, according to the residue normality (Gaussian distribution) and variance homogeneity of each variable. Pearson and Spearman (parametric and no parametric variables, respectively) correlations were used to calculate the relationship between the variables studied in each variable group. Results are reported as untransformed means ± SEM. All values were considered significant at P < 0.05.

Results

Clinical evaluation of lambs showed significant differences in Apgar scores, heart rate (HR) and respiratory rate (RR; Table 1). The preterm group had significantly lower vitality, HR (bradycardia) and RR (bradypnea). However, both groups were normothermic (preterm: 39.93 ± 0.42°C; term: 40.05 ± 0.36°C; P = 0.620) and euglycemic (preterm: 3.34 ± 1.88 mmol/L; term: 2.29 ± 0.82 mmol/L; P = 0.240).

Table 1. Mean ± SE of Apgar score, heart rate and respiratory rate in preterm and term groups.

| Variable          | Groups          | P value |
|-------------------|-----------------|---------|
| Apgar score (0-10)| Preterm 4.00 ± 1.10 | Term 5.83 ± 0.98 | 0.012 |
| Heart Rate (bpm)  | Preterm 99 ± 34  | Term 160 ± 50  | 0.034 |
| Respiratory Rate (mpm) | Preterm 13 ± 10  | Term 47 ± 18  | 0.002 |

At birth, preterm lambs had a low blood pH, indicating acidosis (Table 2). Both groups had high pCO2 values, but there was a severe hypercapnia in the preterm group, indicating a respiratory imbalance. In addition, preterm newborns had hypoxemia, with significantly lower pO2 and SO2 values. There were no significant differences in TCO2 between groups (Table 2). For both groups, the bicarbonate levels remained within normal ranges (Vannucchi et al., 2012; Table 2). However, base excess was altered in both groups, indicating metabolic imbalance at birth (Table 2).

Although no difference was verified between groups, blood sodium concentration (Na+) was at low range (hyponatremia) only for preterm Group (Table 2). On the other hand, blood potassium concentration (K+) remained at normal range for both groups (Vannucchi et al., 2012; Table 2).

Regarding lambs oxidative profile, no difference (P > 0.05) on serum SOD, GPx and TBARS were verified among experimental groups (Table 3). There was a negative correlation (r = -0.914; P = 0.029) between glycemia and pH for the preterm group. Taking into account the oxidative profile of both groups, GPx results negatively correlated with Apgar score (r = -0.753; P = 0.004), HR (r = -0.576; P = 0.005), SO2 (r = -0.634; P = 0.026) and pO2 (r = -0.622, P = 0.030).

Table 2. Mean ± SE of arterial blood gas values, sodium (Na+) and potassium (K+) of preterm and term groups.

| Variable          | Groups          | P value |
|-------------------|-----------------|---------|
| pH                | Preterm 7.07 ± 0.10 | Term 7.27 ± 0.10 | 0.020 |
| pCO2 (mmHg)       | Preterm 85.52 ± 18.65 | Term 45.3 ± 12.62 | 0.001 |
| pO2 (mmHg)        | Preterm 10.67 ± 5.65 | Term 35.67 ± 16.18 | 0.011 |
| SO2 (%)           | Preterm 6.17 ± 5.85 | Term 47.00 ± 28.48 | 0.016 |
| TCO2 (mmol/L)     | Preterm 27.67 ± 1.97 | Term 22.00 ± 5.97 | 0.069 |
| HCO3- (mmol/L)    | Preterm 25.40 ± 1.98 | Term 20.73 ± 5.80 | 0.110 |
| BE (mmol/L)       | Preterm -3.50 ± 3.02 | Term -5.00 ± 6.36 | 0.613 |
| Na+ (mmol/L)      | Preterm 129.20 ± 40.26 | Term 143.20 ± 5.34 | 0.436 |
| K+ (mmol/L)       | Preterm 4.72 ± 0.76  | Term 5.20 ± 2.08  | 0.611 |

pO2: oxygen pressure; pCO2: pressure of carbon dioxide; TCO2: total carbon dioxide; HCO3-: bicarbonate; BE: base excess; SO2: oxygen saturation.

Table 3. Mean ± SE of serum thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) and glutathione peroxidase (GPx) of preterm and term groups.

| Variable | Groups          | P value |
|----------|-----------------|---------|
| TBARS (ng/ml) | Preterm 113.75 ± 19.82 | Term 106.12 ± 15.68 | 0.464 |
| SOD (U/ml)  | Preterm 0.41 ± 0.36  | Term 0.47 ± 0.14  | 0.703 |
| GPx (U/ml)  | Preterm 14.19 ± 4.57 | Term 9.38 ± 3.04 | 0.058 |

Discussion

The Apgar score as an indicator of vitality in the immediate neonatal period may vary among domestic species (Vannucchi et al., 2015b). Lambs born at term in eutocia have Apgar score of approximately 6 immediately at birth (Vannucchi et al., 2012). Thus, based on our results, it is clear that prematurity in sheeps entails the reduction of clinical vitality at birth, since all lambs had clinical depression. Additionally, the preterm group had significantly lower Apgar score, indicating an inadaptability condition of preterm lambs.
during transition to extra uterine life. As a compensatory response to moderate hypercapnia during normal labor, term neonates present increasing heart and respiratory rates (Alonso-Spilsbury et al., 2005). In contrast, the preterm lambs in this study had low HR and RR at birth. Moreover, the premature group (135 days of pregnancy) had severe bradycardia and bradypnea. During neonatal period, the neurological control of cardiovascular system is incomplete due to partial sympathetic nervous activity of the myocardium. Thus, episodes of bradycardia are mostly derived from prolonged hypoxemia (Grundy, 2006). Hence, bradycardia in neonates of the preterm group indicates increased hypoxic stress in comparison to the term group in which lamb respond to the hypoxic stress of labor by increasing their heart rate. These results highlight the low ability of premature lambs to overcome the challenge of lambing.

Premature lambs had blood acidosis, as well as severe hypercapnia. Similar results were obtained previously by Dani et al. (2009), also for preterm lambs. The acid base imbalance that involves primarily changes of blood CO2 has a respiratory origin, causing respiratory acidosis or alkalosis (Houpert, 1996). On the other hand, it is known that premature lambs are relatively tolerant to hypercapnia (Strand et al., 2003). Thus, it is possible to infer that the severity of the acid-base imbalance is not primarily related to hypercapnia, but to the concurrent hypoxia. Therefore, we can assume that the main medical procedure to premature lamb’s resuscitation is to provide adequate pulmonary oxygenation.

In the present study, the preterm neonates had severe hypoxemia at birth, with low values of pO2 and SO2 in comparison to full-term lambs (Vannucchi et al., 2012). Neonatal blood pO2 is influenced by the concentration of inspired oxygen and respiratory rate (Vaala et al., 2006). However, lambs became clinically depressed and bradypneic. Hence, low pO2 values indicated impairment of aerobic activity, which leaded to the onset of anaerobic metabolism. In addition, we observed a negative correlation between glycaemia and blood pH in the Premature Group, which means that consumption of glucose is necessary to achieve an blood acid-base balance, notably through the mobilization of energy reserves in order to increase respiratory and heart rate and compensate the initial hypoxia. Hence, it is extremely important to analyze the metabolic status of premature lambs and proceed to an adequate treatment of hypoglycemia whenever necessary.

The acid-base disturbance of non-respiratory origin should be evaluated by the deficit or base excess (BE) and the concentration of bicarbonate ions (HCO3-; Russell et al., 1996). Our results show that there is no depletion of serum bicarbonate and BE changes at birth, even in preterm lambs. Thus, blood gas alterations at birth reflected the hypoxia of lambing and not neonatal metabolic activity. Regardless of the fact that intrapartum hypoxia is considered physiologic, lambing assistance is of utmost importance, especially for premature lambs prone to more intense clinical depression and blood gas imbalance.

At late gestation, there is an increase of over 150% of the concentrations of SOD, GPx, catalase and glutathione reductase enzymes, parallel to functional maturation of human fetal lungs (Davis and Auten, 2010). Consequently, there is a reduction of cellular lipid peroxidation (Qanungo and Mukherjea, 2000). SOD catalyzes the reaction in which two O2 molecules form H2O2 and O2. Subsequently, H2O2 is metabolized by reduced glutathione (GSH) in H2O and oxidized glutathione (GSSG), catalyzed by GPx (Nichi et al., 2007). In human fetuses, during fetal-neonatal transition and in the immediate postpartum period, there is an increase in the activity of all enzymes that comprise the redox reaction of glutathione, especially GPx and glutathione transferase (Vento et al., 2003). However, in our study, no significant difference in serum GPx and SOD was verified between premature and term lambs. Such results do not indicate improvement in the neonatal enzymatic antioxidant capacity during late gestational period in sheep. Nevertheless, additional experiments are needed using cytosol of erythrocytes to accurately detect neonatal antioxidant profile, because of the higher enzyme concentration compared to plasma (Vento et al., 2003). On the other hand, Frosali et al. (2004) did not find significant differences in SOD and GPx between preterm and term infants, using the cytosol of erythrocytes as biological matrix.

In the present study, we demonstrated a negative correlation of GPx with the Apgar score, HR, pO2 and SO2. Based on such results, we can infer that neonates clinically depressed, in hypoxia and low O2 consumption produce high amounts of ROS that has to be counteracted by GPx. Hence, this enzyme is intensely consumed in such clinical situation. In fact, Vannucchi et al. (2015a) also attested the co-participation of GPx in the correction of neonatal acid-base imbalance in premature puppies. In addition, the action of GPx in reducing the rate of production of new free radicals also played a role in lamb’s vitality, as shown by Apgar score and HR.

Inder et al. (1994) documented increased lipid peroxidation and reduced GPx activity in preterm infants, as well as in low birth weight full-term babies with chronic lung disease. In our work, there was no difference in TBARS values between premature and term lambs. However, we cannot rule out local pulmonary increase in lipid peroxidation of premature sheep, as high production of free radicals is necessary to change serum concentration of TBARS (Ballagipordany et al., 1991). Thus, it is possible that the local pulmonary measurement of TBARS, SOD and GPx over time in neonatal lambs can show differences in redox balance between preterm and term lambs.

To our knowledge, no previous reports have been performed to verify the effects of prematurity on lipid peroxidation and antioxidant enzymes, blood gases and clinical outcome of neonatal lambs. In summary, our data show that preterm neonates are less adapted to the odds of labor and to overcome the immediate changes of extra-uterine life. Furthermore, we verified an influence of glutathione peroxidase in controlling oxidative stress, which highlights mature enzymatic mechanisms of cell redox, even in premature lambs.
This research offers understanding of the medical needs of prematurity, especially regarding acid-base adjustment and mechanical ventilation. However, further studies are necessary to better understand the local pulmonary antioxidant profile of ovine neonates and ultimately establish the mechanisms by which lambs neutralize the increase in reactive oxygen species.

Disclosure statement

The authors report no conflicts of interest.

Authors' contributions

LCGS conceived and performed clinical and laboratorial analysis and drafted the manuscript; FMR and CFL performed clinical and laboratory analysis and the presentation of data, CBF and CIV coordinated and contributed to analysis and discussions and CIV drafted the manuscript. All authors approved the final manuscript.

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