

Energetic metabolic profile of ewes presenting low body condition score induced to subclinical hypocalcemia in early postpartum

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ABSTRACT. The aim of the present study is to assess plasma concentrations of metabolites related to energy balance in ewes showing low body condition score (BCS) induced to subclinical hypocalcemia in early postpartum. Sixteen crossbred ewes (Ideal x Corriedale) presenting BCS <3 were divided in two groups: 1) control group (n = 9), which received no treatment, and 2) hypocalcemia group (n = 7), which was subjected to twelve hours of induced subclinical hypocalcemia through intravenous Na₂EDTA infusion six hours postpartum. Ionized calcium levels were monitored and kept between 0.62 and 0.87 mmol/L. All ewes were subjected to daily blood sampling for five days in order to set the postpartum metabolic profile. There was no interaction between day and treatment in total and ionized calcium concentrations ($P > 0.05$), whereas the mean concentrations of these marks after five days were lower in the hypocalcemia group ($P < 0.05$). However, neither the mean group were different at glucose, non-esterified fatty acids, beta-hydroxybutyrate and insulin ($P > 0.05$) level. These results evidence that, despite the subclinical hypocalcemia induction at early postpartum, ewes presenting low body condition do not change the concentrations of energy balance-related metabolites in the following five days.

Key words: subclinical hypocalcemia, metabolic profile, calcemia, low body condition, sheep.

INTRODUCTION

The late gestation period in sheep is critical, since it demands greater nutritional needs and nutrient availability to udder development and body maintenance (El-sherif and Assad 2001). Pregnant and lactating ewes redirect most glucose and amino acid supplies to the fetal-placental unit and mammary gland throughout this period, since such transfer largely accounts for the high energy requirement (Chilliard 1999). Late gestation and early lactation coincide with a period of low native forage availability in the extensive pasture systems of Southern Brazil (Ribeiro *et al* 2004, Carvalho and Bartello 2009). The low temperatures and high animal stocking per hectare set high metabolic challenges to pregnant ewes, because such factors lead to low energy intake during high-demand periods.

These events, cause gluconeogenesis substrate reduction and lead to hypoglycemia. The decrease of glucose level fosters increased lipolysis rates, as well as the release of fat, triglycerides and NEFA (Harmeyer and Schulunbhon 2006). Such low supply and high nutritional demand increase the risk of developing metabolic diseases. Pregnant and lactating ewes can develop ketosis, which is usually responsible for significant losses in production systems, depending on NEFA mobilization intensity, and the production and metabolization ability of ketone bodies

(Henze *et al* 1998). Negative energy balance intensity also depends on the ability of tissues and their time to adapt to different metabolic pathways adopted for energy production (Chilliard *et al* 2000, Loor *et al* 2006). Smith and Sherman (1994) suggest that ewes must keep a body condition score (BCS) between 3.0 and 3.5 in the final third of pregnancy, 3.5 at birth, and between 2.0 and 2.5 at weaning in order to achieve energy balance.

Besides ketosis, hypocalcemia also contributes to higher production losses and ewe mortality rates during peripartum (Mavrogianni and Brozos 2008). Unlike hypocalcemia in cows, which often happens in postpartum, when it comes to sheep the disease develops from several weeks before birth until the first two weeks after birth. Approximately 60% of the fetal growth takes place in the final gestation period (Twardock *et al* 1973), when the fetus retains higher calcium levels for skeletal growth, providing a negative late pregnancy calcium balance in most sheep (Braithwaite *et al* 1970), which is characterised by total calcium blood levels below 2.0 mmol/L (Oetzel 2004, El-Khodery *et al* 2008). Studies have shown that approximately 50% of pregnant ewes present total calcium concentration in blood plasma below the reference value for late pregnancy, whereas healthy non-pregnant ewes show total Ca plasma concentration close to 1.25 mmol/L (Henze *et al* 1994).

Endogenous glucose production can be compromised during negative energy balance periods, when the excess of ketone bodies are associated with low calcium levels (Schlumbohm and Harmeyer 2003). Furthermore, clinical hypocalcemia can also reduce insulin secretion caused by calcium participation in the insulin exocytosis process in pancreatic β cells (Rutter *et al* 2006), despite the high plasmatic glucose levels (Blum *et al* 1972, Henze *et al* 1998).

Accepted: 02.11.2017.

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Based on these evidences, this study hypothesizes that ewes with low body condition at lambing which are submitted to subclinical hypocalcemia in the postpartum period have greater negative energy balance. The aim of the current study was to assess the effect from induced subclinical hypocalcemia on the energetic metabolic profile of ewes presenting low body condition score during the five first lactation days.

MATERIAL AND METHODS

The Ethics Committee on Animal Experimentation of the Federal University of Pelotas approved all the procedures carried out during the experiment.

The experiment was conducted at the Sheep Experiment Department of NUPEEC - *Núcleo de Pesquisa, Ensino e Extensão em Pecuária* (Livestock Research, Teaching and Extension Center) of the Federal University of Pelotas, Rio Grande do Sul State, Brazil (31° 48'58 "S" and 52° 25'55 W). Sixteen crossbred ewes (Ideal x Corriedale) averaging 3.5 years of age, between the 110th and 140th gestation day, showing body condition score (BCS) lower than 3 (range 1-5) were used (Russel *et al* 1969). During daytime, ewes were kept for six hours in 2-hectare pasture area containing ray grass (*Lolium multiflorum*) and oat (*Avena strigosa*). The pasture area was divided in alternated paddocks, according to forage availability to minimize eventual nutritional management effects. The estimated total forage availability during the experimental period was 1,100 kg dry matter per hectare. Samples were collected for pasture bromatological analysis through the manual simulated-grazing method. The recorded means were 20.2%, for crude protein; 25%, for acid detergent fiber (ADF); and 60.5%, for neutral detergent fiber (NDF).

Sheep at the lambing were randomly divided in two groups: 1) control (n=9), and 2) hypocalcemia (n=7). Group 2 (hypocalcemia) consisted of sheep subjected to twelve hours of induced subclinical hypocalcemia six hours after lambing. Each ewe gave birth to a single lamb. Mean lamb body weight was not different between groups (3.1±0.7 vs 4±0.4 Kg/BW for the control and hypocalcemia groups, respectively). Subclinical hypocalcemia induction was conducted by administrating 5% Na₂EDTA (pH 7.35-7.45) through intravenous catheter at rate 10 drops/minute, according to Jørgensen *et al* (1999). The administration rate depended on the ionized calcium plasma level, which was measured every one hour in order to be kept between 0.62 and 0.87 mmol/L. The 5 mL blood aliquot was directly collected from the catheter with the aid of a heparinized syringe (2% heparin) aiming at monitoring the ionized calcium. The samples were analysed in the Orion 290A + pH pH advanced ISE7MVT (Thermo Fisher Scientific, USA) meter adapted with a selective electrode for calcium ions. Sheep were kept with their offspring to prevent further stress throughout the induction period.

Blood samples were collected in a daily basis for five postpartum days. The first collection (day 1) was performed 12 hours after lambing. Blood samples were collected through jugular vein puncture according to the Vacutainer system (BD Diagnosis, Sao Paulo, Brazil) by using two previously identified tubes. One tube contained anticoagulant (10% EDTA) and antiglycolitics (12% KF Potassium fluoride) for glucose analysis. The other one did not have anticoagulant and was used for calcium, magnesium, phosphorus, chloride, albumin, insulin, NEFA and β-hydroxybutyrate analysis. Blood samples were centrifuged right after collection to obtain serum or plasma, which were placed in microtubes and frozen for further analysis.

Calcium, magnesium, phosphorus, chloride, glucose and albumin levels were evaluated from the first day until the fifth day after subclinical hypocalcemia induction, and were analysed by colorimetric enzymatic kits (Labtest Diagnostica SA, Brazil), according to the instructions of the manufacturer, in the UV/visible spectrophotometer (FEMTO 435, FEMTO Industry and Trade Instruments, São Paulo, Brazil).

Insulin levels were evaluated from the first to the third day after induction subclinical hypocalcemia. The analysis was performed using a kit DSL-10-1600 ACTIVE Insulin sandwich immunoassay kit (Diagnostics Systems Laboratories Inc., Webster, TX, USA), according to the method described by Lee *et al* (2002). Readings were conducted in plate reader (Thermo Plate TP-Reader, São Paulo, Brazil).

The NEFA levels were evaluated from the first to the fifth day after subclinical hypocalcemia induction, the analysis was performed by using the RH Series NEFA kit (WakeChemicals, USA); the β-hydroxybutyrate analysis was conducted with the Ranbut commercial kit (Randox Laboratories, Oceanside, CA, USA). Both analyses followed the method described by Ballou *et al* (2009).

STATISTICAL ANALYSIS

Statistical analysis was performed by SAS statistical software (SAS Institute Inc., Cary, NC, USA), version 9.0. Results were presented as mean and standard error of the mean; data were analysed by through ANOVA with repeated measurements; collection time was the repetitions, sheep were the experimental units, animals within their respective groups were the random effect, and the groups were the fixed effect. Blood levels of the analysed metabolites were included as dependent variables. Means were compared through Tukey-Kramer test. Statistical differences were defined as $P \leq 0.05$.

RESULTS

Figure 1 shows that hypocalcemia was successfully induced, since the calcium concentration was kept in rates

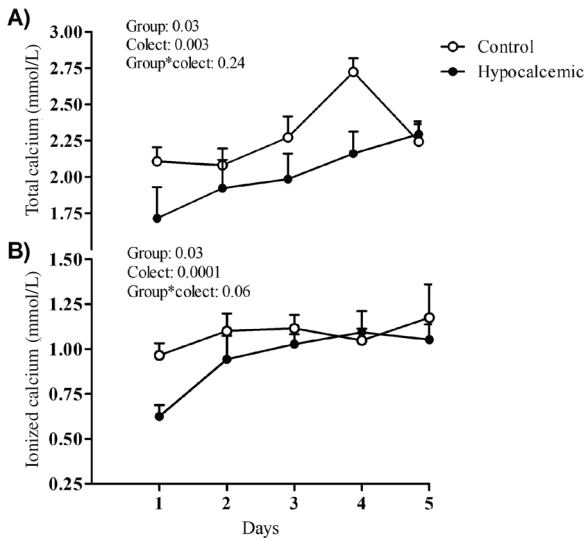


Figure 1. Total (A) and ionized calcium (B) levels (in serum and plasma, respectively) in the control (○, n=9) and hypocalcemic (●, n=7) groups during the 5-day postpartum period.

lower than 2 mmol/L of total calcium and between 0.62 and 1 mmol/L of ionized calcium in the hypocalcemia group from day one. Apparently, the ionized calcium levels did not influence just induction day when their means were 0.96 ± 0.07 mmol/L and 0.62 ± 0.06 mmol/L, in the control and hypocalcemic groups, respectively. Mean ionized calcium was different between the control

and hypocalcemic groups ($P=0.03$) during the 5-day period (1.10 ± 0.04 and 0.97 ± 0.04 mmol/L, respectively). The same was observed for total calcium (figure 1) level during this period, the recorded means were 2.29 ± 0.06 and 2.01 ± 0.07 mmol/L for the control and hypocalcemic groups, respectively ($P=0.03$). Induced-hypocalcemia ewes maintained total calcium levels below 2.1 mmol/L in the first three postpartum days. The levels of energetic metabolics (figure 2) were not affected ($P>0.05$), despite the occurrence of subclinical hypocalcemia. The mean NEFA concentration recorded for the control group was 0.98 ± 0.17 mmol/L, and 1.00 ± 0.15 mmol/L ($P=0.85$) for the hypocalcemia group. The mean BHBA concentration for the control group was 0.83 ± 0.23 mmol/L, and 0.71 ± 0.16 mmol/L ($P=0.55$) for the hypocalcemia group. Glucose was within the physiological level (figure 2) during the early lactation period, with values of 2.99 ± 0.22 mmol/L and 2.91 ± 0.20 mmol/L, in control and hypocalcemic groups, respectively ($P=0.96$). The concentration of this metabolic was different between collections only, the highest concentrations were observed at the first postpartum day. The mean insulin concentration recorded for the control group was 4.81 ± 0.45 μ UI/mL, and 3.15 ± 0.62 μ UI/mL ($P=0.06$) for the hypocalcemia group.

Apparently, subclinical hypocalcemia induction influenced the phosphorus levels ($P=0.002$) and led to higher serum levels in hypocalcemic ewes after five days (hypocalcemic group 1.74 ± 0.12 mmol/L; control group 1.39 ± 0.08 mmol/L) (table 1), thus showing greater amplitude at the

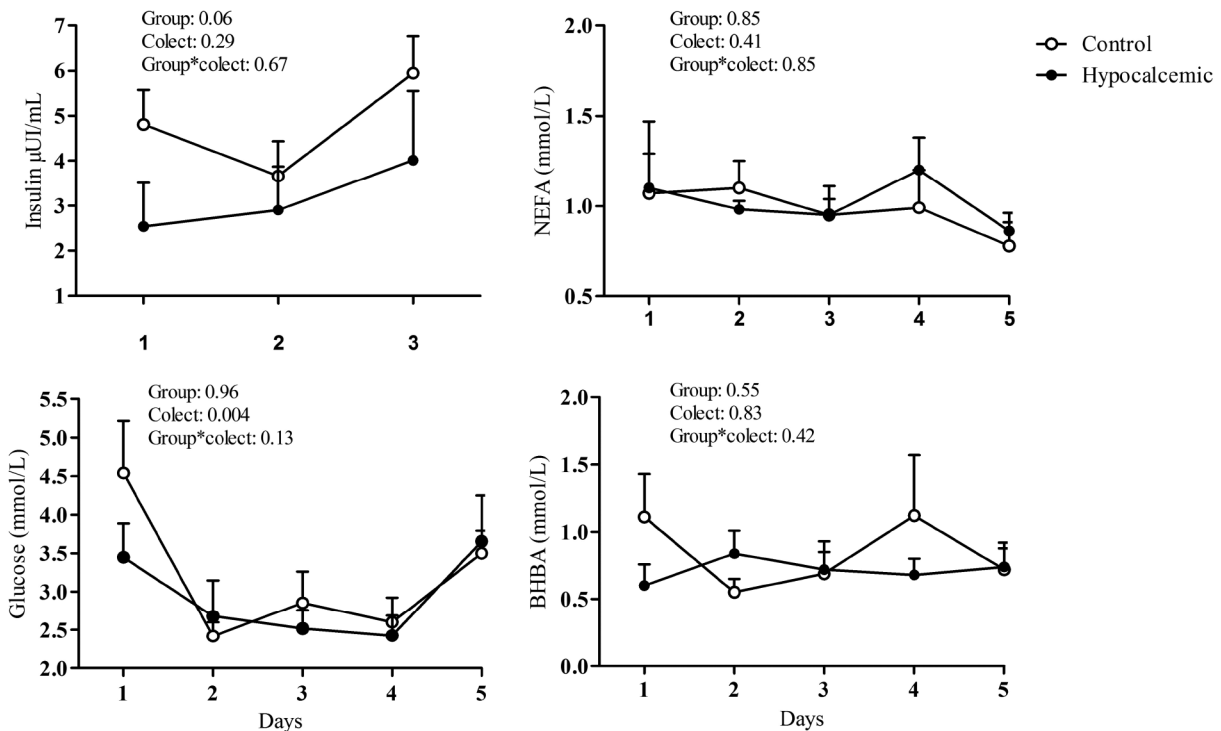


Figure 2. Insulin, (μ UI/ml), non-esterified fatty acids (mmol/L), glucose (mmol/L) and β -hydroxybutyrate (mmol/L) levels in the 5-day postpartum period for the control (○, n=9) and hypocalcemic (●, n=7) groups.

first day. The concentration levels of albumin, magnesium and chloride in the serum were not affected by subclinical hypocalcemia (table 1, $P>0.05$).

DISCUSSION

Figure 1 showed that subclinc hypocalcemia induced at the first postpartum day can disrupt calcium metabolism in the following days. Although renal excretion of the CaEDTA complex is completed few hours after Na_2EDTA infusion (Aronson and Ahrens 1971), in some cases hypocalcemia is induced with Na_2EDTA , and it leads to lower gastrointestinal tract motility. Such low motility impairs the animals' recovery ability, because the calcium absorbed by the intestine is essential to the maintenance of the calcium level itself (Jørgensen *et al* 1999). Thus, calcium level recovery in ewes subjected to subclinical hypocalcemia induction would happen slowly. This recovery process can be followed by lower dry matter intake due to decreased gastrointestinal motility (Goff 2006). However, the induced subclinical hypocalcemia did not influence energy balance in the present study (figure 2). The NEFA level in both groups was consistent with the catabolism state (Caldeira 2005). Firat and Özpınar (2002) measured the NEFA level in ewes with adequate body condition and reported levels varying from 0.64 to 0.68 mmol/L at the 100th gestation day and at the 10th lactation day, respectively. However, Cal-Pereyra *et al* (2015) observed NEFA level 1.28 mmol/L in ewes restricted to the last gestation, whereas sheep without restrictions presented values close to 0.18 mmol/L. Food restriction also led to glucose level reduction right after the beginning of the restriction period in the study by Cal-Pereyra *et al* (2015). However, significant differences in glucose levels were observed only from the 54th hour on, between animals with and without restrictions, and the physiological level returned during the 102nd hour after. In addition, glucose level reduction was followed by NEFA and BHBA increase in the circulatory starting from the 48th hour of the restriction periods. Such

levels remained high up to the 144th hour, and the animals were not monitored after this period.

Despite the low body condition at lambing and the high NEFA levels in the current study, the mean BHBA levels remained within the baseline limits for the period (Durak 2006, Harmeyer and Schlumbohm 2006). Ewes seem to have optimized the use of ketone bodies by peripheral tissues in order to supply energy during the pre-partum period. It may be the consequence from a strategy developed to adapt to low volatile fatty acids and glucose availability (Caldeira 2005). The same condition was also observed during postpartum, regardless of the group. Although some sheep showed ketone body peaks, such peaks did not meet the hypocalcemia period, therefore, it may have contributed to the absence of effects on glucose metabolism. Glucose was within the physiological level throughout the early lactation period (Caldeira 2005, Roubies *et al* 2006). The highest concentrations were observed at the first postpartum day, and it can be attributed to cortisol actions. This hormone gradually increases five days before delivery, and allows greater circulating glucose availability; thus, it influences the action of glucose carrier GLUT4 in peripheral tissues, as well as stimulates hepatic gluconeogenesis (Bassett 1963, Fowden 1997, Ingvarsten and Andersen 2000). The results contradict the reports of Schlumbohm and Harmeyer (2003), who showed the hindering effect of hypocalcemia on glucose turnover. However, the study by Schlumbohm and Harmeyer (2003) proved that ewes were in good body condition before the beginning of the experiment. These authors evaluated sheep in different production stages and suggested a sequence of events that take place in the body of hyperketonic and hypocalcemic sheep, namely: decreased endogenous glucose and restricted glucose availability, lipolysis rate stimulation, and increased BHBA rate with consequent glucose depression intensification. Such effect was not observed in cases in which the calcium rate dropped down without parallel BHBA increase. Subclinical hypocalcemia may have significant depressor effect on carbohydrate metabolism

Table 1. Some metabolic parameters of the hypocalcemic and control groups in 5-day postpartum period (mean + standard deviation).

Variable	Postpartum days										Value P		
	Control					Hypocalcemic					Group	Colect	Group* Colect
	1	2	3	4	5	1	2	3	4	5			
Phosphorous (mmol/L)*	1.40±0.14	1.39±0.26	1.58±0.18	1.33±0.23	1.27±0.07	2.02±0.35	1.50±0.26	1.49±0.0.19	1.78±0.20	1.87±0.27	0,002	0,89	0,46
Magnesium (mmol/L)	0.94±0.11	0.89±0.12	0.93±0.08	0.68±0.07	0.67±0.15	1.01±0.16	1.05±0.43	1.03±0.26	0.72±0.07	1.18±0.32	0,14	0,45	0,71
Chlorides (mmol/L)	106.81±2.84	110.30±5.74	104.65±4.18	102.76±2.51	103.73±2.49	111.05±4.85	110.27±3.69	115.51±4.53	108.57±4.30	107.36±4.77	0,29	0,50	0,46
Albumin (g/L)	28.75±0.76	29.10±1.19	28.57±1.06	27.19±1.06	27.77±1.09	30.16±3.17	28.72±1.72	30.13±1.90	28.88±1.29	28.68±1.23	0,39	0,74	0,94

* Variables with the means from all the experimental period differed between groups ($P<0.05$).

when ewes are not adapted to energy deficit and to high levels of ketone bodies. Insulin concentrations were not influenced by hypocalcemia, although studies in dairy cattle already showed that low calcium concentrations can decrease the secretion of this hormone (Blum *et al* 1972); this effect seems to be restricted to clinical hypocalcemia (total calcium <1.25 mmol/L).

Serum albumin was within the reference range for the species in both groups, when protein metabolism was assessed (Balıkcı *et al* 2007). However, due to the low synthesis and degradation rates of this protein, longer deficiency periods (approximately one month) are necessary, so that blood concentrations could drop down (Contreras *et al* 2000). Seemingly, subclinical hypocalcemia induction influenced the phosphorus levels and led to higher serum levels in hypocalcemic ewes at the end of five days, thus showing greater amplitude at the first day. This outcome may have resulted from the phosphorus release from bone tissue and mediated by the PTH action, in order to increase calcium levels. Although this hormone also increases phosphorus excretion in the proximal convoluted tubule, the presence of calcium in the tubule lumen is a mechanism used to stimulate phosphorus absorption, which may be a decisive factor for hypocalcemia induction (Bisello and Fiedman 2008). Magnesium and chloride levels were not affected by subclinical hypocalcemia (table 1).

Although subclinical hypocalcemia may be identified as a disease contributing to negative energy balance in the postpartum of ruminants (Goff 2006, Schlumbohm and Harmeyer 2003), the herein recorded metabolic profile suggested that these effects were not observed in ewes presenting low body condition at lambing. Therefore, it suggests that animals adapt to this condition, and that such adaptation is mainly based on glucose, NEFA and ketone body availability and supply (Herdt 2000).

Based on results, which were in opposition to the hypothesis addressed in the present study, it is possible to conclude that subclinical hypocalcemia induction during early postpartum does not change the concentrations of metabolites related to energy balance of ewes presenting low body condition during the first five lactation days.

ACKNOWLEDGEMENTS

The authors would like to thank CAPES - Coordination for the Improvement of Higher Education Personnel - for their financial support.

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