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Subclinical ovine gestational toxaemia at parturition: Influence on determinant variables in lamb survival

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ABSTRACT - The objective of this study was to determine the impact of metabolic changes occurring in induced subclinical gestation toxaemia in ewes on metabolic parameters and vigour in the first 72 h of their lambs' life. Fifty-one adult Corriedale ewes of known gestation date and fed on a natural pasture were randomly divided into four groups at day 145 of gestation. Group A (ewes with single pregnancies) and group C (ewes with twin pregnancies) were fed on a natural pasture throughout the trial; group B (ewes with single pregnancies) and group D (ewes with twin pregnancies) were subjected to 75% feed restriction for three days until they reached glycaemia and β-hydroxybutyrate (BHB) values indicative of subclinical gestational toxaemia. The birth-to-first station and birth-to-first suckling times of the born lambs were studied. Within the first hour after parturition and at 24, 48, and 72 h, glycaemia and BHB concentrations were determined in the blood of lambs, and body temperature and body weight were assessed at the same time. Relative weight gain at 72 h of age was calculated for all lambs. The BHB concentration determined at all times studied showed no difference among the groups. Subclinical gestation toxaemia induced in ewes subjected to feed restriction causes a decrease in the glycaemia of their lambs in the first hour of life; however, it does not cause an increase in ketone bodies. The metabolic changes induced in the mothers do not have negative effects on the vigour of the lambs at birth, as the weight, temperature, and the time it took for lambs to stand and suckle are not affected. However, they have a negative effect on relative weight gain in the critical period of the first 72 h of life.

Keywords: ewe, lamb, pregnancy, restricted feeding, vigour

1. Introduction

Ovine gestational toxaemia is a metabolic pathology that occurs in the last six weeks of gestation (Moghaddam and Hassanpour, 2008; Cal-Pereyra et al., 2015) due to the inability to maintain energy homeostasis, resulting from the increased glucose requirements of the foetus(es) at this time, causing a negative energy balance (Schlumbohm and Harmeyer, 2008; Brozos et al., 2011; Santos et al., 2011). Lambs of ewes suffering from the clinical form of this disease have significantly higher mortality in the neonatal period of the first 72 h of life, varying from 30 to 70% (Miodovnik et al., 1982). They are often stillborn, and if born alive, they are usually small, premature, underweight, weak, dying within a few days of birth (Constable et al., 2017; Marutsova and Marutsov, 2017). These mothers also produce little milk (Miodovnik et al., 1982; Brozos et al., 2011; Barbagianni et al., 2015b), making their lambs susceptible to hypothermia and diarrhoea (Cal Pereyra et al., 2011). It is also believed that lamb mortality is partly due to foetal ketoacidosis (Miodovnik et al., 1982).

The subclinical form of the disease is characterised by steadily increasing hypoglycaemia and hyperketonemia in dams in the absence of clinical signs (Barbagianni et al., 2015b; Cal-Pereyra et al., 2015; Marutsova and Marutsov, 2017). At the blood level it can be diagnosed when glycaemia values are between 24.29 and 32.95 mg/dL at which time the serum concentration of β-hydroxybutyrate (BHB) is between 1.23 and 3.29 mmol/L (Abreu-Palermo et al., 2021). However, the effect of subclinical gestational toxaemia on lamb survival is not known.

The objective of this study was to determine the impact of metabolic changes occurring in induced subclinical gestational toxaemia in ewes on metabolic parameters and vigour in the first 72 h of life of their lambs.

2. Material and Methods

The research protocol was carried out in Libertad, San José, Uruguay (34°38' S; 56°39' W), and was conducted according to the institutional committee on animal use (PI 13/14 - Exp 111130-000636-14).

We used 51 adult Corriedale pregnant ewes (28 carrying single foetuses and 23 twins) with a body condition above 2.5, rated on a range of 0 to 5 (Russel et al., 1969), with known pregnancy date, and fed on natural pasture. On day 145 of pregnancy, they were randomly divided into four groups, two groups carrying a single foetus (A and B) and two groups carrying twins (C and D).

Considering that the gestation length of Corriedale ewes is 147.9±1.9 days (Benech, 2007), the following protocol was applied from that moment on:

Groups A (n = 13) and C (n = 12) (control groups): ewes in these two groups were fed until lambing on natural pasture.

Groups B (n = 15) and D (n = 11): from day 145 of gestation, the ewes of these two groups were confined in roofed pens with concrete floors. During confinement, they were subjected to a gestational toxaemia-inducing environment, which consisted of acute feed restriction for a maximum of three days, with free access to water. Each ewe in group B was fed 0.4 kg/day of alfalfa hay (equivalent to 25% of daily requirements, 0.89 Mcal of metabolizable energy [ME]), while each ewe in group D was fed 0.525 kg/day of alfalfa hay (equivalent to 25% of daily requirements, 1.22 Mcal of ME) (AFRC, 1993).

At the time when ewes in groups B and D reached glycaemia values indicative of subclinical gestational toxaemia (28.62±4.33mg/dL; Cal Pereyra et al., 2015), they were removed from feed restriction and switched to pasture feeding with natural pasture (Abreu-Palermo et al., 2021).

The gestation length in ewes was evaluated in days (expressed in mean ± SEM), obtaining: group A: 148.3±0.7; B: 148.1±0.3; C: 147.8±0.3; and D: 148.1±0.3 days. And the number of lambs born per group was: A = 13; B = 14; C = 19, and D = 19.

2.1. Determinations in ewes

Blood samples were obtained by jugular vein puncture with 10-mL syringes and 18 G needles. All ewes were bled at 145 days gestation and at 12, 24, and 48 h after the start of feed restriction to determine glycaemia. On day 145 of pregnancy and at 24 and 48 h after the start of the trial, β-hydroxybutyrate (BHB) was also determined.

Glycaemia was determined by an enzymatic colorimetric method, using Glucose Liquicolor® Commercial Kit (Human, Germany), measuring absorbance at 500 nm at 37 °C with the HUMALYSER Junior digital colorimeter. β-hydroxybutyrate was determined by an enzymatic colorimetric method using Ranbut® Commercial Kit (Randox Laboratories Ltd., United Kingdom), and absorbance was determined at 330 nm at 37 °C with the HUMALYSER Junior digital colorimeter.

2.2. Determinations in lambs

Blood measurements of glycaemia and BHB of lambs in all groups were performed within the first hour of life (immediately after feeding) and at 24, 48, and 72 h post parturition. At the same time, the rectal temperature of all lambs was determined with a digital thermometer, body weight was recorded with a digital scale, and the relative weight gain of lambs was calculated according to the following equation (Cal-Pereyra et al., 2011):

$$\frac{\text{Live weight at 72 h (kg)} - \text{Live weight at birth (kg)}}{\text{Live weight at 72 h (kg)}} \times 100$$

Immediately after lambing, the birth-first station time (time in minutes between birth and the moment the lamb first succeeded in standing on its four limbs) and the birth-first suckling time (time in minutes between birth and the moment the lamb first succeeded in sucking colostrum) were recorded for each lamb (Capper et al., 2006; Benech, 2007).

2.3. Statistical analysis

Normality of the different variables was determined using Shapiro-Wilk tests. Serum levels of glycaemia and BHB in ewes and lambs, as well as weight in lambs, were normally distributed and analysed using the following fixed effect model:

$$Y_{ij} = \mu + T_i + e_{ij},$$

in which Y_{ij} is the response variable, μ is the overall mean, T_i is the fixed effect of treatment (Group A, B, C, D), and e is the residual error.

The significances of these variables between groups were analysed by one-way ANOVA followed by Scheffe's test.

Significances of glycaemia and BHB at the different extraction times (1, 24, 48, and 72 h) within the same group were analysed by ANOVA followed by Scheffe's test for repeated samples.

Birth-first station and birth-first suction times and temperature had a non-parametric distribution, and were evaluated by the Kruskal-Wallis test.

Statistical analyses were performed using STATA 15.2 (Statistics/Data Analysis. StataCorp LLC). Significant differences were considered when $P<0.05$.

3. Results

3.1. Metabolic parameters in ewes

At day 145 of gestation, prior to the start of feed restriction, glycaemia concentration did not show significant differences among the experimental groups (A = 50.53±1.68 mg/dL; B = 55.13±2.14 mg/dL, C = 57.17±2.67 mg/dL, and D = 51.27±2.73 mg/dL). The glycaemia concentration of ewes in groups B (single gestation) and D (twin gestation) decreased 12 h after the start of feed restriction ($P<0.001$), reaching glycaemia values indicative of subclinical gestational toxaemia 48 h after the start of treatment (B = 30.67±2.37 mg/dL and D = 28.40±3.39 mg/dL) (Table 1; Abreu-Palermo et al., 2021).

At day 145 of gestation, before starting feed restriction, BHB concentration did not show significant differences among experimental groups (A = 0.69±0.05 mmol/L, B = 0.47±0.07 mmol/L, C = 0.72±0.14 mmol/L, and D = 0.85±0.20 mmol/L). The serum BHB concentration of ewes in the groups subjected to feed restriction (groups B and D) showed an increase (P = 0.0001) 24 h after starting the treatment, reaching serum values of this ketone body indicative of subclinical gestational toxaemia 48 h after starting the treatment (B = 1.87±0.12 mmol/L and D = 2.21±0.42 mmol/L) (Table 1; Abreu-Palermo et al., 2021).

Table 1 - Mean±SEM blood β-hydroxybutyrate (BHB) and glucose concentrations in ewes under feed restriction and ewes fed in natural field, from day 145 of pregnancy and at 12, 24, 36, and 48 h after the treatment started (Abreu-Palermo et al., 2021)

	Glycaemia (mg/dL)				BHB (mmol/L)			
	A	B	C	D	A	B	C	D
Day 145	50.53±1.68	55.13±2.14A	57.17±2.67A	51.27±2.73A	0.69±0.05A	0.47±0.07A	0.72±0.14	0.85±0.20A
12 h	53.00±3.84	46.93±2.31	56.33±1.83a	41.72±2.26b	-	-	-	-
24 h	49.83±1.19	40.93±2.08	56.00±3.05a	37.40±4.30b	0.55±0.08a	1.52±0.14b	0.67±0.09a	2.17±0.24c
36 h	-	33.69±2.18	-	29.60±4.20	-	-	-	-
48 h	44.69±2.75a	30.67±2.37Bb	40.83±1.60B	28.40±3.39B	0.34±0.04Ba	1.87±0.12Bb	0.63±0.05a	2.21±0.42Bb

SEM - standard error of the mean.
Group A: single-bearing ewes fed *ad libitum*; group B: single-bearing ewes under feed restriction; group C: twin-bearing ewes fed *ad libitum*; and group D: twin-bearing ewes under feed restriction.
A,B - Statistical differences between times within the same study group found with Student's test (P<0.05), in the column, are represented by different uppercase letters following mean values for each time.
a,b,c - Statistical differences among study groups for each time, found with ANOVA and Sheffe's test (P<0.05), are represented by different lowercase letters following mean values.
The absence of uppercase or lowercase letters implies the lack of significant differences among times or groups.

3.2. Metabolic parameters in lambs

Within the first hour of birth, the glycaemia of single lambs born to dams fed *ad libitum* prior to parturition was higher (P = 0.016) than that of single lambs born to dams with feed restriction (Table 2). Higher glycaemia concentration (P = 0.011) was observed within the first hour of parturition among lambs born to the two control groups (groups A and C) (Table 2). However, within the first hour postpartum, there was no significant difference in the glycaemia of twin-born lambs (groups C and D). At 24 h after birth, lambs in all experimental groups showed a significant increase in glycaemia (P<0.001). This was maintained during the following 48 and 72 h, with no statistical difference among groups at any of these times.

The BHB concentration determined within the first hour after parturition, as well as that recorded 24, 48, and 72 h after the birth of the lambs, did not show any difference among groups.

Table 2 - β-hydroxybutyrate (BHB) and blood glucose (mean±SEM) concentrations in lambs born to fed-restricted and pasture-fed dams at 1, 24, 48, and 72 h after parturition

Time (h)	Glycaemia (mg/dL)				BHB (mmol/L)			
	A	B	C	D	A	B	C	D
1	68.3±8.5aA	41.07±5.7bA	41.3±3.8bA	49.3±4.2abA	0.11±0.03	0.08±0.05	0.16±0.05	0.08±0.01
24	105.4±8.4B	96.93±9.8B	98.6±7.9B	95.7±7.8B	0.33±0.04	0.29±0.04	0.21±0.03	0.27±0.02
48	114.8±6.1B	105.36±6.9B	96.92±13.2B	115.3±5.1B	0.33±0.04	0.35±0.04	0.18±0.03	0.23±0.04
72	120.4±4.2B	106.42±8.7B	97.5±10.1B	121.5±6.78B	0.47±0.04	0.43±0.07	0.49±0.08	0.49±0.11

SEM - standard error of the mean.
Group A: lambs born single to ewes fed *ad libitum*; group B: lambs born single to ewes under feed restriction; group C: twin lambs born to ewes fed *ad libitum*; and group D: twin lambs born to ewes under feed restriction.
A,B - Statistical differences (P<0.001) between times within the same experimental group found with ANOVA and Sheffe's test.
a,b - Statistical differences (P<0.05) between experimental groups for each time found with ANOVA and Sheffe's test.

No differences were found in parturition-to-first station times between lambs born single (groups A and B) and between lambs born twins (groups C and D). However, twin lambs in the control group took less time to stand than single-born lambs in the control group (P = 0.0412).

There was also no difference between single-born and twins in the time taken for effective suckling (Table 3).

The temperature values of the lambs in all groups did not differ in any of the samples recorded (Table 3).

Weight recorded within 1 h of birth and at 24, 48, and 72 h did not differ between single-born lambs or twin lambs (Table 3). However, within the first hour after birth, when observing the lambs of the control groups, the lambs of group A were heavier (P<0.001). This difference was maintained at 24, 48, and 72 h postpartum (P<0.001, respectively) (Table 3). Lambs in group A gained 14.01% live weight at 72 h, with no weight loss (showing the lowest coefficient of variation). However, lambs in the groups subjected to feed restriction had the highest coefficient of variation in weight gain at 72 h (Table 4). Lambs in groups B and D showed mean gains of 5 and 3.8% and weight losses of up to 18 and 18.3%, respectively. Lambs in the twin control group showed an average gain of 6.6%, losing up to 10% weight over the studied period (Table 4).

Table 3 - Body weight (g; mean±SEM) and temperature of lambs (°C) at 1, 24, 48, and 72 h after birth from ewes from the different experimental groups

	Experimental group			
	A	B	C	D
n	13	14	19	19
Body weight (g)				
1 h	4969±171a	5093±184a	3884±120b	4154±137ab
24 h	4815±217a	5014±167a	3741±115b	4107±146ab
48 h	5263±249a	5292±190a	3822±123b	4236±163ab
72 h	5900±244a	5523±188a	4134±118b	4441±173ab
Temperature (°C)				
1 h	40.3 (36.2–40.9)	40.2 (39.3–41.0)	39.8 (39.1–40.6)	40.1 (38.3–41.6)
24 h	39.7 (37.7–40.6)	39.6 (36.3–40.5)	39.5 (38.9–40.6)	39.6 (39.0–40.3)
48 h	40.0 (37.5–40.7)	40.0 (39.9–40.6)	39.5 (38.9–40.5)	39.6 (38.3–40.8)
72 h	39.9 (39.7–40.5)	39.9 (37.7–40.8)	39.5 (39.7–40.5)	39.9 (37.7–40.8)
Birth-to-first station (min)	25.0 (10–64)a	18.0 (13–60)ab	14.0 (2–57)b	15.0 (1–53)ab
Birth-to-first suckling (min)	45.0(20–92)	49(26–112)	36.0(16–161)	38.0(6–147)

n- number of lambs; SEM- standard error of the mean.
Group A: lambs born single to ewes fed *ad libitum*; group B: lambs born single to ewes under feed restriction; group C: twin lambs born to ewes fed *ad libitum*; and group D: twin lambs born to ewes under feed restriction.
Birth-to-first station and birth-to-first suckling times of the born lambs expressed as median (p5-p95).
a,b - Statistical differences between experimental groups for each time found with ANOVA and Sheffe's test (P<0.05).
The absence of uppercase or lowercase letters implies the lack of significant differences among times or groups.

Table 4 - Relative weight gain (mean±SEM) at 72 h of age of lambs, expressed as percentage values, maximum, minimum, and coefficient of variation (CV)

	A	B	C	D
Percentage (%)	14.01±2.12	5.22±3.26	6.64±1.98	3.79±2.62
Maximum (%)	24.28	24.59	18.18	20.46
Minimum (%)	4.76	−18.00	−10.25	−18.31
CV (%)	48	217	130	294

Group A: lambs born single to ewes fed *ad libitum*; group B: lambs born single to ewes under feed restriction; group C: twin lambs born to ewes fed *ad libitum*; and group D: twin lambs born to ewes under feed restriction.

4. Discussion

Considering that glycaemia and BHB values reached 48 h after the start of feed restriction in ewes of groups B and D, and that none of these animals showed clinical signs of the disease, we can assume that these ewes were undergoing subclinical gestational toxaemia (Abreu-Palermo et al., 2021).

Adequate energy reserve at parturition is significant for neonatal survival and resistance to adverse climatic conditions (Cal-Pereyra et al., 2011; Vannucchi et al., 2012). In addition, Banchero et al. (2010) and Cal-Pereyra (2007) suggested a direct relationship between maternal and foetal plasma glucose.

The low glycaemia values recorded during the first hour of life in lambs born to ewes in groups B and D reflected those their mothers presented 48 h after the start of feed restriction. This scenario was reported by Bell and Greenwood (2016) and Dreyer (2012), who proposed that maternal nutritional deficiency will produce foetal hypoglycaemia.

However, Vannucchi et al. (2012) proposed that hypoglycaemia occurs in the first hour of the lambs' life, caused by the drastic reduction of foetal circulation as a consequence of parturition, which is associated with a rapid depletion of hepatic glycogen and inefficient glucose homeostasis. Furthermore, the same authors found no relationship between maternal and neonatal glycaemia, which agrees with the glycaemia found in the lambs of group C, whose mothers did not receive feed restriction. However, the condition of twin lambs may explain the lower glycaemia concentration found concerning the single lambs of group A.

In this trial, the glycaemia values recorded in the lambs within 1 h of parturition agreed with those reported by Vannucchi et al. (2012), who considered concentrations of 46.86±21.87 mg/dL as physiological. Furthermore, the glycaemia concentrations recorded in the lambs of the four experimental groups from 24 to 72 h after birth coincided with those reported by Silva et al. (2018), who suggested that the elevation of glucose was the result of the contribution of this metabolite through colostrum suckling.

Concerning litter size, the lower glucose values obtained in twin lambs could be explained by Dutra and Banchero (2011), who proposed that these differences might be due to placental efficiency, since in twin ewes, the surface area of exchange of this organ decreased with litter size. Rumball et al. (2008) stated that placental mass per foetus was reduced by about one-third in twins compared with singletons, although the total placental mass was higher. Therefore, the maternal-foetal glucose gradient was smaller in twins, suggesting reduced placental glucose consumption or increased efficiency of placental glucose transfer. Therefore, these authors suggested that the relative reduction in placental size in those lambs could be associated with compensatory changes in placental function, ensuring an adequate supply of substrate to the foetus.

There is insufficient literature on the placental transfer of ketone bodies and their metabolic effect on the ovine foetus (Miodovnik et al., 1982). These authors reported that there was a placental transfer of BHB to the foetus, stating that the high concentrations in the maternal uterine vein produced an increase in the blood concentration measured in the foetal carotid arteries, although the values detected in the latter were very low (0.15±0.03 mmol/L) (Miodovnik et al., 1982). These authors reported similar values to those obtained in the present experiment at the time of parturition in all experimental groups.

Palacín et al. (1984) demonstrated that the arterial blood concentration of BHB in the ewe was higher than that of the foetus, suggesting that in those situations in which there was an increase in the maternal concentration of ketone bodies, the concentrations of this metabolite in the foetus were of less importance. Ovine placenta is classified as epitheliochorial, as it has six layers, which condition the permeability to specific metabolites (Roa et al., 2012; Furukawa et al., 2014). This fact would explain why no relationship was observed between the high levels of ketone bodies in feed-restricted dams and the low concentrations of this metabolite in their lambs at the time of parturition.

The BHB concentration observed in the four experimental groups as the days passed could be due to the production of this ketone body at the hepatic level, caused by the mobilisation of lipid and glycogen stores. In addition, heat production is stimulated after birth by the combustion of brown fat, with a transition from net intrauterine anabolism to catabolism to meet the dramatic increase in oxygen consumption at birth (Symonds et al., 1995).

The time it takes for lambs to stand and suckle determines their survival (Dwyer, 2008; Dutra and Banchero, 2011). It was reported that, physiologically, lambs could stand within 30 min of birth (Dwyer, 2003; Banchero et al., 2010; Stevenson, 2014), consistent with those times obtained in this trial.

After lambs can stand, they seek the udder to suckle. Several authors reported that this event occurred around 2 h after birth in both single and twin births, which was consistent with the times obtained in this experiment in all experimental groups (Dwyer, 2003; Banchero et al., 2010; Stevenson, 2014).

It has been shown that single-born lambs, being heavier, took less time to stand than lambs born as twins. Owens et al. (1985) stated that the larger the litter, the longer the time lambs needed to stand and suckle, finding that lambs with higher birth weights stood and reached the udder faster than lighter lambs. The data obtained in our experimental conditions did not agree with these reports.

In this work, the lambs of all groups maintained a stable body temperature in the first 72 h of life. The recorded temperature values were within the physiological parameters for lambs during the critical period of 72 h post-parturition (Dwyer and Morgan, 2006; Vannucchi et al., 2012). The maintenance of homeothermy expressed in this work was necessary for the lambs to express their normal standing and suckling behaviour within established physiological ranges, maintaining a positive vicious circle in favour of survival (Dwyer and Lawrence, 2005; Dwyer, 2008).

The average weight values obtained in this experiment for single and twin lambs were within the ranges cited in the literature as optimal for their survival (Banchero et al., 2005; Stevenson, 2014; Barbagianni et al., 2015a) for the Corriedale breed under pastoral conditions (Benech, 2007). Montossi et al. (2005) and Dalton et al. (1980) suggested that the optimal birth weight range was between 3.5 to 5.5 kg. Weights below 3 kg and above 5.5 kg decreased the probability of survival (Ganzábal, 2005; Dwyer, 2008; Dreyer, 2012; Stevenson, 2014). In this experimental trial, there were no differences in the weights of lambs born to dams subjected to feed restriction and those born to pasture-fed dams, when comparing groups of single lambs (A and B) with each other and twin lambs with each other (C and D). It should be noted that the feeding restriction was carried out close to lambing, a situation that did not allow changes in lamb weights at birth.

Single and twin lambs born to dams with feed restriction at the end of gestation had lower relative weight gain at 72 h of life and higher weight losses. It has been shown that, in ewes poorly nourished during the last weeks of gestation, udder weights and udder development were reduced, leading to delayed initiation of lactation, with reduced prenatal colostrum accumulation and milk production during the 18 h after lambing (Banchero et al., 2005; Dwyer, 2008). The strong relationship between nutrition during gestation and early lactation is well documented (Banchero et al., 2005; Dreyer, 2012).

Colostrum accumulation occurs two to three days before lambing (Banchero et al., 2005; Banchero et al., 2010), coinciding with the start of the present trial. In twin ewes, colostrum production could be so diminished that it could not be present at the time of parturition (Banchero et al., 2005), which may explain the relative weight loss in twin lambs born to well fed dams. These authors proposed that the absence of colostrum was attributed to the poor quality or nutritive value of the pasture or a reduction in voluntary intake during the last weeks of pregnancy (Banchero et al., 2005; Harmeyer and Schlumbohm, 2006). In this trial, the twin ewes of the control group were given feed that covered their needs. Therefore, the lamb weight losses could be attributed to the voluntary reduction in their mothers' intake at this stage. Dwyer (2008) suggested that progesterone blocked colostrum synthesis by inhibiting lactose synthesis, which is osmotically active, whose main function is to regulate the water content of milk, determining its viscosity (Banchero et al., 2005). When the lactose content is not sufficient, colostrum viscosity is very high, causing the lamb to expend more energy to suckle an adequate amount of colostrum (Banchero et al., 2005).

5. Conclusions

Subclinical gestational toxaemia induced by feed restriction at the end of gestation decreases glycaemia and increases ketone bodies in the dams. These metabolic changes in the restricted ewes result in a decrease in glycaemia of their lambs in the first hour of life. However, this does not lead to increased ketone bodies in the lambs. The metabolic changes caused by the toxaemia of subclinical gestation at parturition have no adverse effects on the vigour of the lambs at birth. Lamb weight, temperature, and time taken to stand and suckle are not affected. However, relative weight gain is negatively affected in the critical period of the first 72 h of life.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

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