

Faecal thiaminase, plasma lactate and pyruvate concentrations and erythrocyte transketolase activity changes in apparently normal replacement ewes after the initiation to the pasture

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Abstract

A study was made to investigate faecal thiaminase and the thiamine-related biochemical changes in apparently normal replacement ewes with a feed change, after the initiation without adaptation to the new pasture. Twenty-four female ewes were divided into two groups. Group A was managed in a system based on pasture and was compared with group B system based on a diet of concentrate and straw until moving to pasture 9 weeks after. Blood samples for lactate, pyruvate and erythrocyte transketolase activity determinations and faeces for thiaminase estimation were evaluated chronologically. At the end of a 126 days experimental period, live weights of groups were similar. We confirmed that clinically normal sheep may have thiaminase activity in the faeces and concluded that the thiaminase release increased during the diet changes, from concentrate to pasture, and that their continued excretion could develop some degree of thiamine deficiency.

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1. Introduction

Polioencephalomalacia (PEM), also known as cerebrocortical necrosis (CCN), is a common nervous system disorder, which has been frequently reported in ruminants (Chahar et al., 1993; Lonkar and Prasad, 1993; Horino et al., 1994a,b). Despite multiple potential causes, PEM has traditionally been believed to result from thiamine deficiency; by a lack of de novo rumen microbial synthesis, administration of thiamine antagonists, such as amprolium or microbial thiaminases that could destroy thiamine (Horino et al., 1994a; Gould, 1998). Thiamine is essential in the metabolism of carbohydrates. A deficiency of thiamine causes accumulation

of blood pyruvate, lactate and decrease blood transketolase activity (Horino et al., 1994a; Lonkar and Prasad, 1993; Dabak and Gul, 2004).

Results obtained in previous studies on spontaneous cases of PEM in Northeast of Spain had seasonal clustering in the spring in the replacement ewes after being exposed without adaptation to grazing pastures in a traditional system. In all the case flocks, thiaminase was found in the rumen content and faeces of animals autopsied and in the faeces of some or all samples from normal replacement sheep (Ramos et al., 2003).

The association of thiaminase with thiamine deficiency and PEM in ruminants has been firmly established. Cases of PEM have been associated with either the ingestion of feedstuffs containing thiaminase or changes in the ruminal environment which make it more favourable for the production of thiaminases (McGuirk, 1987). However, thiaminase activity has been detected in

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faeces of sheep with PEM and in clinically normal animals in contact with cases of PEM or not history of PEM (Thomas, 1986; Thomas et al., 1987).

The observations made in previous studies on spontaneous cases of PEM in the replacement ewes after being exposed without adequate adaptation to grazing pastures in a traditional system (Ramos et al., 2003) and the lack of systematic reports in order to evaluate the changes of different parameters related with thiamine deficiency on these animals, replacement ewes, lead us to propose this study.

PEM is a thiamine-responsive disease and only young ruminants are affected normally. We wanted to develop the study, under conditions of normal farm management in our area, in which the initiation to pastures takes place between 2 and 6 months of age.

In accordance with those previously explained, the purpose of the present study was to evaluate chronologically the effect of feed changes in the replacement ewes on thiaminase excretion in the faeces, thiamine-related biochemical changes in blood, and live-weight in replacement ewe. This relationship was chronologically evaluated in two groups of animals: group A grazing from 8th to 9th weeks old and group B grazing from 17th to 18th weeks old.

2. Materials and methods

2.1. Animals

A total of 24 female ewes Rasa Aragonesa breed were used in this study. All animals suckled their dams with additional concentrate until weaned at 6 to 7 weeks of age. At this age, animals were separated from their mothers. All replacement ewes were fed with straw and concentrates (composition: 17.5% crude protein, 4.6% crude fibre, 4% crude fat matter, ash 6%). Supplements: copper 6 mg/kg and vitamins A, E, and D), and had unlimited access to water. At 8–9 weeks of age, the animals were divided into two groups with 12 animals in each one. The first group (A group) on by grazed pasture. The second group (B) was housed and fed with the same concentrate and straw during 9 weeks and later was fed for 6 weeks on the same pasturage than group A.

2.2. Samples

Body weight, feed, blood and faeces samples of all animals were obtained at 8 to 9 weeks of age, when the animals were divided into two groups (sample number 0), and 1, 2, 3, 5, 8, 9, 10, 11, 13 and 15 weeks after (samples number 1–10).

Faeces samples were manually collected from the rectum using a new plastic bag for each animal. Faecal egg

counts for parasitological control were developed during the trial and all results were negative. Faeces samples were also stored at -20°C until analysis of thiaminase. Ten pasture subsamples were collected from the grazed area, *Lolium multiflorum* (Italian ryegrass), then pooled and one representative sample was stored at -20°C until analysis. Thiaminase activity was also analysed in concentrates.

Blood samples were collected between 8:00 and 9:00 h a.m. from jugular vein of each animal and were distributed into three tubes for the measurement of erythrocyte transketolase activity (ETKA), lactate and pyruvate concentrations.

For the erythrocyte transketolase assay, the heparinized blood was centrifuged at 1100g for 10 min, plasma and buffy coat layers were removed by suction, and packed erythrocytes were washed three times with a cold isotonic saline solution (9 g NaCl/L). After removal of supernatant, de-ionised distilled water was added in a volume equal to that of the packed erythrocytes to promote osmotic lysis. Storage was kept at -70°C (Anderson and Nicol, 1986).

For lactate estimation, blood was collected in a tube containing fluoride-oxalate as anticoagulant. The sample was mixed well by gentle inversion and placed on ice bath. The plasma was separated within 1 to 2 h of sampling by centrifugation at 400g for 10 min. For pyruvate assay 2 mL of blood was collected and mixed immediately with 4 mL of cold 8% perchloric acid for deproteinization. Samples were centrifuged (1500g for 10 min) to obtain a clear supernatant. The plasma or supernatant was separated within 1 to 2 h of sampling, and stored at -20°C until analysis usually within 4 weeks.

2.3. Laboratory analysis

Total thiaminase activity in feed samples and in faeces from 24 animals was determined using the radioactive method of Edwin and Jackman (1974) and modified by Edwin (1979). The samples were lyophilized and 1 g of faecal material weighed and extracted with a citrate-phosphate buffer (pH 6.4, 0.1 M, 2 mL/g of sample), centrifuged (1500g for 10 min) and 0.5 mL of supernatant used for assay. [Thiazole-2- ^{14}C] thiamin hydrochloride (Amersham International) was used as a substrate. In each analysis, a horsetail (*Equisetum arvense*) sample was treated in the same way and included as control. The thiaminase activity was proportional to the amount of radioactivity extracted and was expressed as units (one unit, 1 U = 1 μmol thiazole/min) per g DM.

For measurement of erythrocyte transketolase activity blood haemolysates were centrifuged at 2000g for 15 min and the supernatant used for the assay. Erythrocyte transketolase activity was determined in the absence

(ETKA) and presence (TPP effect) of added thiamine pyrophosphate by a fluorimetric method according to Anderson and Nicol (1986). Values for non-stimulated and stimulated enzyme activity were obtained by calibration with sedoheptulose-anhydride (Sigma Diagnostic Co., St. Louis, S 3375) treated as if it were haemolysate. Haemoglobin concentration in blood haemolysates was determined using the cyanmethaemoglobin method of Boehringer–Mannheim (Test-Combination Hemoglobina Number 124729).

Lactate estimation was determined using Lactate 735 (Sigma Diagnostic Co., St. Louis, USA) and pyruvate with Pyruvate kit Number 726-UV (Sigma Diagnostics Co., St. Louis, USA). In each analysis for lactate and pyruvate, normal and elevated commercial controls (Sigma Metabolite Controls S 3006 and S 3005) were included.

2.4. Statistical analysis

Differences between groups were calculated by a Kolmogorov–Smirnov nonparametric analysis as well as the median and interquartiles range. Statistical significance was assumed to exist when the probability was <0.05.

3. Results

3.1. Thiaminase activity

The concentrate and pasture samples *L. multiflorum* (Italian ryegrass) had not thiaminase activity. However, all the animals released once at least thiaminase in faeces during the study. In faecal samples, thiaminase activity was between 1.3 and 16.2×10^{-4} U per g DM. In one animal, the enzyme was found in 7 samples of faeces and in two animals in 6 samples, without evidence of clinical abnormality. The faecal thiaminase elimination was not continuous in animals with three or more positive samples. These animals were intermittent excretors.

The highest number of animals excreting thiaminase in both groups was reached 2 weeks after the initiation to the pasture and it gradually diminished. The proportion of animals excreting thiaminase varied from minimum 8.3% (1/12) to maximum 91.6% (11/12) at each group examined (Table 1).

3.2. Plasma lactate and pyruvate concentrations

The values of lactate and pyruvate in the replacement ewes are presented in Table 1. The pattern of plasma lactate was similarly altered as pyruvate concentration. Both concentrations increased throughout the study, however there were significant differences between the two groups. The plasma lactate and pyruvate concentrations in group A were significantly higher ($P < 0.01$)

Table 1

Experimental day/age (week)	Pyruvate ($\mu\text{mol/L}$)		Lactate (mmol/L)		Faecal thiaminase	
	A ($n = 12$), Median (IQR)	B ($n = 12$), Median (IQR)	A ($n = 12$), Median (IQR)	B ($n = 12$), Median (IQR)	A ($n = 12$), (Positive/n)	B ($n = 12$), (Positive/n)
0/8–9	3.64 (2.54)	4.16 (1.75)	0.78 (0.36)	0.70 (0.28)	(2/12)	(1/12)
1/9–10	8.02 (1.75)**	4.38 (2.19)	2.54 (0.71)**	0.78 (0.26)	(2/12)	(1/12)
2/10–11	8.42 (2.39)*	5.09 (2.45)	2.55 (0.76)**	1.16 (0.34)	(9/12)	(2/12)
3/11–12	6.48 (3.57)	7.02 (7.76)	3.05 (1.32)*	1.33 (1.65)	(9/12)	(1/12)
4/13–14	8.89 (2.98)	7.56 (4.83)	2.45 (1.47)	1.80 (1.32)	(6/12)	(2/12)
5/16–17	9.64 (1.82)	8.33 (4.12)	3.01 (1.10)	2.35 (1.02)	(3/12)	(1/12)
6/17–18	10.74 (3.24)	10.48 (2.63)	2.64 (0.37)	2.58 (0.82)	(2/12)	(3/12)
7/18–19	9.43 (1.49)	9.47 (5.48)	2.88 (0.11)	2.81 (2.15)	(3/12)	(11/12)
8/19–20	10.39 (2.46)	9.95 (7.98)	2.59 (0.42)	1.76 (1.44)	(1/12)	(6/12)
9/21–22	8.55 (2.98)	10.78 (5.08)	2.62 (0.75)	1.97 (1.54)	(2/12)	(6/12)
10/23–25	9.87 (2.37)	11.23 (3.86)	2.79 (2.57)	2.29 (0.66)	(2/12)	(3/12)

Values are expressed as median and interquartiles range (IQR).

Significantly different between groups A and B at **($P < 0.01$) or *($P < 0.05$).

than those in group B as early as day 7 after the initiation to the new pasture.

3.3. ETKA, TPP effect and live weight

ETKA, TPP effect, and body weight in the replacement ewes are presented in Table 2. The TPP effect and ETKA changed through the course of the study and no significant differences were found between both groups. ETKA values gradually decreased reaching the lowest level at about half of the study. On the contrary, the evolution of the TPP effect was in reverse order. The highest differences between both groups were found in animals' body weight. These differences were in relation to food change. At the end of the study, both groups had similar body weight.

3.4. Relationship between faecal thiaminase and the other parameters

In order to develop the statistical analysis between the values of lactate, pyruvate, ETKA, TPP effect and the body weight in the last sample obtained, the animals were subdivided into four groups. Groups A1 ($n = 6$) and B1 ($n = 7$) included the animals with one to three thiaminase positive samples faeces and the groups A2 ($n = 6$) and B2 ($n = 5$) the animals with more than three thiaminase positive samples faeces during the trial. The ETKA was depressed ($P < 0.05$) and TPP effect elevated ($P < 0.05$) in erythrocytes from the animals with higher thiaminase frequency of excretion in faeces.

4. Discussion

4.1. Thiaminase activity excretion

The observations of other authors (Thomas et al., 1987) that clinically normal sheep and lambs may have thiaminase activity in the faeces/rumen have been confirmed in the present work. The concentrate and pasture samples had no thiaminase activity. However, it was found that up to 100% of the clinically normal animals may be excreting thiaminase once at least and that over 41.6% of the animals have been thiaminase excretors four or more times during this study. If thiaminase excretion is lengthy continuous, it can be reasonably assumed that the level of the enzyme in the rumen must also be raised and that the animal can suffer some degree of thiamine deficiency. Nevertheless, despite the presence of thiaminase in faeces, no clinical symptoms of thiamine deficiency or PEM were seen in any animal.

The approach used in this study provides a temporal sequence in which changes in faecal thiaminase in both groups were evident as early as day 14 after the initia-

Table 2
Blood, erythrocyte transketolase activity (ETKA), thiamine pyrophosphate (TPP) effect and body weight in animals grazing permanent pasture (group A) and animals first feeding concentrate and pasture later (group B)

Experimental day/age (week)	ETKA (mU/g Hb)		TPP effect (%)		Body weight (kg)	
	A ($n = 12$), Median (IQR)	B ($n = 12$), Median (IQR)	A ($n = 12$), Median (IQR)	B ($n = 12$), Median (IQR)	A ($n = 12$), Median (IQR)	B ($n = 12$), Median (IQR)
0/8–9	945 (186)	948 (134)	14.5 (18.8)	15.3 (6.3)	15.5 (3.4)	15.2 (3.3)
1/9–10	819 (169)	861 (310)	22.0 (3.9)	18.0 (7.8)	15.9 (3.7)	16.4 (3.1)
2/10–11	660 (460)	710 (376)	39.0 (40.0)	32.0 (48.0)	16.3 (2.9)*	19.2 (3.2)
3/11–12	613 (578)	544 (385)	28.3 (56.7)	35.2 (28.6)	19.0 (3.1)**	23.3 (3.1)
4/13–14	576 (438)	495 (128)	37.9 (48.0)	42.5 (28.5)	21.5 (2.9)**	26.4 (3.3)
5/16–17	527 (505)	504 (314)	42.5 (98.0)	48.0 (58.0)	26.2 (2.3)**	32.3 (3.7)
6/17–18	395 (455)	477 (149)	48.0 (124)	59.5 (47.0)	27.0 (3.1)**	35.2 (4.9)
7/18–19	458 (473)	469 (489)	56.5 (52.0)	76.5 (92.0)	26.8 (2.8)**	33.1 (4.5)
8/19–20	541 (563)	643 (346)	32.0 (87.0)	27.0 (20.0)	28.3 (4.1)	32.0 (3.4)
9/21–22	696 (361)	666 (275)	33.5 (38.0)	28.5 (26.0)	29.4 (4.1)	31.0 (2.8)
10/23–25	681 (203)	707 (213)	28.0 (23.0)	24.0 (24.0)	30.4 (4.7)	30.9 (2.8)

Values are expressed as median and interquartiles range (IQR).

Significantly different between groups A and B at **($P < 0.01$) or *($P < 0.05$).

tion of transition to the grazing diet. The changes in the diet probably acted as a primary factor for microbial production of thiaminase. These results confirm the previously obtained observation on spontaneous cases of PEM in replacement ewe after grazing pastures (Ramos et al., 2003) and agree with a case of PEM Ben Said et al. (1986) where the animals developed clinical signs 10 days after the commencement of feeding on a new growth pasture.

4.2. Lactate and pyruvate concentrations and ETKA and TPP effect

Lactate and pyruvate concentrations were within normal range (Scott, 2002; Dabak and Gul, 2004), although there were significant differences between the two groups. Lactate and pyruvate concentrations in replacement ewe from group A were significantly higher ($P < 0.01$) than group B as early as day 7 after the initiation to the pasture. The blood lactate or pyruvate concentrations may be more affected by changes in the diet of group A after the initiation to the pasture opposite to group B fed with concentrates, than low thiamine status. In addition, no significant differences between groups were found in the other biochemical indicators of thiamine deficiency: ETKA and TPP effect.

The highest TPP effect and ETKA values were formed at the beginning of the trial. ETKA and TPP effect were changing through the course of the study and no significant differences appeared between the two groups. ETKA and TPP effect has been widely used as an index for detecting thiamine inadequacy in ruminants and also PEM by many authors (Lonkar and Prasad, 1993; Thomas et al., 1990).

4.3. Relationship between faecal thiaminase and ETKA, TPP effect and live weight

The animals with more than three samples thiaminase positive faeces during the trial had significant lower ETKA ($P < 0.05$) and higher TPP effect ($P < 0.05$) in the last sample obtained. The presence of thiaminase in the faeces may be associated with impaired thiamine metabolism (Edwin et al., 1976; Thomas, 1986) but it is important to emphasize that a single estimation of faecal thiaminase may lead to overestimate the thiaminase status of animals.

Although the TPP effect is an indicator for thiamine deficiency, there are not enough data concerning the quantitative relationship between the TPP effect and the incidence of PEM in ruminants. In young ruminants, the TPP effect above 45% indicates thiamine deficiency (Blazovsky et al., 1980). In sheep, considered as normal, the TPP value should be up to 25%, whereas animals with PEM the mean was 71% (Bogin et al., 1985). In goats with PEM, TPP values means were 54%, whereas

normal goats have 47% of TPP (Thomas et al., 1987). We found an increase in the TPP effect, with values above 45% in apparently normal sheep from both groups and higher values of TPP in animals with more than three thiaminase positive samples faeces during the trial. In other papers, the TPP effect has been associated with reduced growth rates in sheep (Thomas, 1986), however, it has not been possible to show this influence in our study.

4.4. Conclusions

We can conclude that the faecal thiaminase release of replacement ewes may increase during the changes in the diet and that continuous faecal thiaminase excretion can allow to identify periods during which animals could develop some degree of thiamine deficiency. However, it is somehow difficult to apply in clinical practice.

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