Effect of the feeding pattern on rumen wall morphology of cows and sheep

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Abstract

Nutritional manipulation of the rumen wall volatile fatty acid absorption capacity can be a strategy to control ruminal acidosis in dairy cows. Aiming to induce morphological rumen wall variation through diet and to establish efficient papillae morphological markers 2 experiments were performed. In experiment 1, seven rumen-cannulated cows were fed with concentrate 1 or 4 times a day for 19 days followed by fasting for 72 hours. Ruminal papillae were collected on days, 0, 4, 12 and 19 of the treatment period, and 24, 48 and 72 hours after onset of fasting which was able to induce papilar involution. Lower concentrate feeding frequency was associated to insulin increasing over time ($P=0,02$) and higher ($P=0,03$) mitotic index (MI), but it did not affect other morphological parameters. In experiment 2, two non-simultaneous trials with 3 rumen-cannulated ovines in each, were conducted and animals were fasted abruptly for 72 hours after feeding. Papillae were collected at the end of the feeding period and at the end of the 72 hour fasting period. MI was higher in the feeding period than the in fasting period ($P<0,01$), but other morphological parameters were not able to respond to nutritional variation. Among the morphologic markers studied MI seems to be the best variable for evaluation of the rumen epithelium morphologic response to feeding plans. Frequency of concentrate feeding may be used to regulate rumen papillae morphology.

Introduction

High producing cows are usually switched from forage to grain-rich diets at calving, as a way of matching energy consumption to the high metabolic demand of lactation. Ingestion of large amounts of carbohydrate rich foodstuffs may result in excessively high ruminal volatile fatty acid (VFA) concentration and low pH. Ruminal acidosis may adversely affect animal performance and health through its detrimental effects on ruminal motility[1,2], fiber fermentation[3], feed intake[4] and rumen wall morphology[5]. Dietary manipulation of rumen papillae size may be able to increase the efficiency of milk production by allowing high levels of energy absorption at low ruminal concentration of VFA. Papillary size can be associated to absorption capacity[6,7,8]. Grain feeding during late gestation stimulated papillary proliferation before calving in dairy cows[6], because VFA stimulate ruminal ephitelium prolipheration by direct[9] and/or by a mediated insulin indirect effect[10,11]. Furthermore the temporal pattern in rumen VFA production may affect the response in rumen epithelial cell growth[9,12]. Increasing the fractional rate of VFA absorption by feeding energy rich diets pre-calving requires the consideration that reduced dry matter intake is observed at the time of calving[13]. Low intake around calving time may result in acute depression in rumen wall absorptive capacity, counteracting dry cow feeding practices designed to increase ruminal VFA clearance

post calving. Decreased feed intake in sheep had an acute depressive effect upon the capacity of the rumen wall to absorb VFA[14].

Morphological markers for ruminal papillae are necessary to evaluate potential mucosa absorption capacity in induction studies of rumen wall VFA absorption capacity. Morphological markers must be correlated with absorption capacity and be sensible to nutritional changing in an acute way as much as possible aiming to reduce the duration of the experiments. Frequently a simultaneous effect of diet on papillar size and rumen absorption capacity was not observed[14]. Imprecise techniques for rumen absorption capacity or morphological evaluations could explain this fact.

The objective of this experiment was to evaluate acute morphological response of the bovine and ovine ruminal epithelium to changes in the feeding plan aiming to define useable morphological markers in nutritional manipulation of VFA rumen wall absorption capacity studies. We also aimed to determine if a low daily frequency of concentrate feeding, capable of inducing rumen VFA and plasma insulin spikes, is a plausible strategy to manipulate rumen papillae morphology in cows. Fasting after inducing papillae proliferation was done to simulate the period of intake depression observed in periparturient dairy cows.

Material and Method

Experiment 1:

Seven non-lactating rumen-cannulated cows, 4 Jerseys and 3 Holsteins (mean body weight of 375 and 527 kg, respectively), were blocked by breed and randomly assigned to one of two treatments after a 7-day standardization period feeding on Coastal bermudagrass hay (93% dry matter, 6.7% crude protein and 87.1% neutral detergent fiber on a DM basis) at *ad libitum* intake. Cows were housed individually in sand bedded stalls and provided continuous access to feed and water. During the 19-day comparison period, cows were offered Coastal *ad libitum* and consumed 0,5% of body weight in concentrate either once (T1), at 6:00 o'clock, or four times (T4) a day, at 6:00, 12:00, 18:00, and 24:00 hours. Concentrate composition on an as fed basis was 90% ground corn, 6% soybean meal, 2% urea, and 2% minerals. A 72-hour fasting period, simulating a period of extreme intake depression near calving time was started on day 20 of the comparison period.

On day 7 of the standardization period 12 papillae were randomly collected by biopsy from the cranial sac of the rumen (*Atrium ruminis*) to be used as covariate in the model for analysis of variance. Twelve rumen papillae were also collected by biopsy on days 4, 12 and 19 of the comparison period and 24, 48, and 72 hours after the onset of the fasting period. Biopsies were always performed at 12:00 o'clock and were obtained from approximately 10 cm medial from the left rumen wall and 5 cm cranial from the cranial pillar. Collected papillae were kept on a PBS solution pH 7.4 (0.790 g NaCl, 0.223 g Na_2HPO_4, 0.052 g NaH_2PO_4, 100 ml H_2O), until and during scanning (Scanjet 4 C, Hewlett Packard®) for macroscopic measurement (Figure 1). Papillae length, area, and the area over length was determined by subjecting scanned images to the image analysis software Sigma Scan Pro 2.0 (Jandel Corporation®). The twelve papillae were fixed in Bouin liquid for 22 hours, dehydrated in ethanol solutions, diphanized in xylene (Dimethylbenzene – $C_6H_4(CH_3)_2$), included in paraffin , and 5 mm sections stained with haematoxylin and eosin. The mitotic and apoptotic index of the cells of the basal layer of the epithelium was determined in each papillae with an optic microscope (Ken-a-Vision®) at 400 magnification. All nucleus with mitotic or apoptotic figures were counted and each of them were expressed as a percentage of the visible nucleus. The percentage of cells undergoing mitosis or apoptosis was the mean of two independent evaluators for each animal within sampling day. In addition other microscopic measurements were done through an

micrometric ocular lens. For papillae width and connective tissue width determination of the papilla tip was used as reference point. These variables were determined at 5 and 10 µm from the tip and the average of these two points was considered as papilla width or connective tissue width. A papilla piece between 5 and 15µm from the tip was considered to measure papilla epithelial pegs. The number of epithelial pegs was counted on both sides of the papilla in this piece and the average of both was considered as the papilla epithelial peg number.

Blood samples were obtained from the blood of the caudalis mediana vein at 0, 90, 180 and 360 minutes after the 6:00 o'clock hay feeding on day 7 of the standardization period or concentrate on days 4, 12, and 19 of the comparison period. During the fasting period one plasma sample was obtained at 6'00 o'clock. Samples were collected with 5 ml capacity evacuated blood collection tubes, with 0.05 ml of EDTA (Venoject® - Terumo®). Blood was centrifuged at 2118 x g for 15 minutes and the plasma frozen at –20 ºC until analysis. Plasma insulin was determined by radioimmunoassay using a Coat-a-Count Kit® as modified by Vaughn et al[15].

Samples of rumen fluid were obtained concurrently with plasma samples. A 100 ml glass cup was capped with the hand and opened in the rumen ventral sac. Samples were immediately strained through 4 layers of cheesecloth and the pH measured with a Schott Geräte® ion analyzer using a general-purpose combination electrode.

Plasma insulin and rumen pH data during the comparison period was analyzed using the repeated measure approach of the Mixed procedure of SAS[16]. The structure of covariance used was the one with the largest value for the Akaike's information criterion. Covariance structures considered were compound symmetric, autoregressive of the first order, and unstructured. The following model was used: $Y_{ijkw} = \mu + V + B_i + F_j + D_k + T_w + F_jD_k + F_jT_w + D_kT_w + F_jD_kT_w + e_{ijkw}$, where: μ = Overall mean, V =

covariate (measurement of the same variable during the last day of the standardization period), B_i = breed effect (i = Jersey, Holstein), F_j = frequency of concentrate feeding effect (j = 1 per day, 4 per day), D_k = sampling day effect (k = 4, 12, 19), T_w = time post feeding effect (w = 0, 90, 180, 360), F_jD_k, F_jT_w, D_kT_w, $F_jD_kT_w$ = two and three term interactions, e_{ijkw} = residual error. A cow within the treatment was used as the error term to test the frequency of concentrate feeding effect. To analyze plasma insulin and rumen pH including the fasting period data, the average of the four post feeding times at each sampling day of the comparison period was used and the single daily observation of the three fasting days. These and papillae morphology data were analyzed using the repeated measures approach with the following model, also using cow within frequency of concentrate feeding to test the frequency of concentrate feeding effect: $Y_{ijk} = \mu + V + B_i + F_j + D_k + F_jD_k + e_{ijk}$, where: μ = Overall mean, V = covariate (measurement of the same variable during the last day of the standardization period), B_i = breed effect (i = Jersey, Holstein), F_j = frequency of concentrate feeding effect (j = 1 per day, 4 per day), D_k = sampling day effect (k = days 4, 12, and 19 of the comparison period and 24, 48, and 72 hours of fasting), F_jD_k = interaction of frequency of concentrate feeding and sampling day, e_{ijk} = residual error.

Experiment 2:

Six rumen-cannulated sheep divided in two trials were used. In the first trial the animals were fed corn grain to meet maintenance requirements and alfalfa silage *ad libitum* for 21 days followed by a 72 hour fasting period. In the second trial 3 sheep were fed *ad libitum* with totally mixed ration which had 48% alfalfa and 52% corn grain for 21 days followed by a 72 hour fasting period. The goal of the fasting period, as in experiment one, was to induce ruminal epithelium acute morphological response. Ruminal papillae were collected by biopsy

Table 1 – Plasmatic insulin, ruminal pH, mitotic and apoptotic index, papillae macroscopic and microscopic measurements of non-lactating and non-pregnant cows fed with concentrate 1 or 4 times a day and Coastal bermudagrass hay for 19 days followed by a 72 hour fasting period in experiment 1. Means adjusted for the same variable value at the end of the standardization period. Lavras, 2004.

	1	4	SE	P		
				F[1]	D[2]	T*D[3]
Insulin (μUI/ml)	13.6	11.8	1.8	0.54	<0.001	0.02
pH	7.05	7.09	0.11	0.84	<0.001	<0.001
Papillae macroscopic width - Area/length (mm)	1.52	1.67	0.14	0.52	<0.001	0.72
Total papillae microscopic width (μm)	98.9	103.2	13.4	0.86	0.23	0.42
Papillae tissue connective width (μm)	69.5	78.0	10.5	0.65	0.17	0.34
Number of papillae epithelial pegs	6.7	7.8	1.0	0.55	0.41	0.63
Mitotic index (% of basal nucleus)	0.97	0.75	0.03	0.03	<0.001	0.49
Apoptotic index (% of basal nucleus)	0.41	0.40	0.08	0.95	0.01	0.11

1- Statistical P values for feeding frequency effect.
2 - Statistical P values for Day effect.
3 - Statistical P value for frequency*day interaction effect.

Table 2 – Microscopic measurements, mitotic and apoptotic index of ovine ruminal papillae feeding and fasting in the experiment 2. Lavras, 2004

	Feeding	Fasting	SE	P
Total papillae microscopic width (μm)	27.7	32.3	2.4	0.23
Papillae tissue connective width (μm)	7.6	11.7	2.3	0.26
Number of papillae epithelial pegs	20	22.9	1.2	0.14
Mitotic index (% of basal nucleus)	0.73	0.48	0.04	<0,01
Apoptotic index (% of basal nucleus)	0.29	0.33	0.02	0.29

Figure 1 - Scanned ruminal papillae biopsied from cranial sac on fourth day of the comparison period and digitalized in natural size

from the rumen cranial sac (*Atrium ruminis*) on day 21 of the feeding period and at the end of the 72 hour fasting period. Histological procedure and measurements, mitotic and apoptotic index determination were done as was described for experiment 1. Data were analyzed for the GLM procedure of SAS according to the following model: $Y_{ijk} = \mu + S(P)_i + P_j + T_k + e_{ijk}$, where: μ = Overall mean, $S(P)_i$ = Sheep in the trial (i = 1 to 6), F_j = Trial effect (j = 1 to 2), T_k = Treatment effect (k = feeding or fasting), e_{ijk} = residual error.

Results and Discussion

Acute response on the mitotic index was observed according to changes in feeding plans in experiment 1 and 2 (Table 1 and 2). Mitotic index showed to be a good variable to express short changes in feeding plans such as concentrate feeding frequency as well as big changes like fasting. Because of its high statistical significance, especially in experiment 2, the mitotic index seems to be an efficient variable for morphological evaluations of ruminal epithelium when few experimental units are used like in VFA absorption experiments. Acute response to nutritional changes has been observed by several authors. Tamate et al.[17] observed a ruminal basal layer cell degeneration after 3 days of fasting and cellular proliferation response after 6 hours re-feeding. Circadian cycle of ruminal papillae cell growth was different in ovines feeding one or two times a day[18]. Nutritional changes seem to induce a quick response in ruminal epithelium cell growth. After feeding VFA peaks occur in the rumen and insulin peaks in blood plasma. Volatile
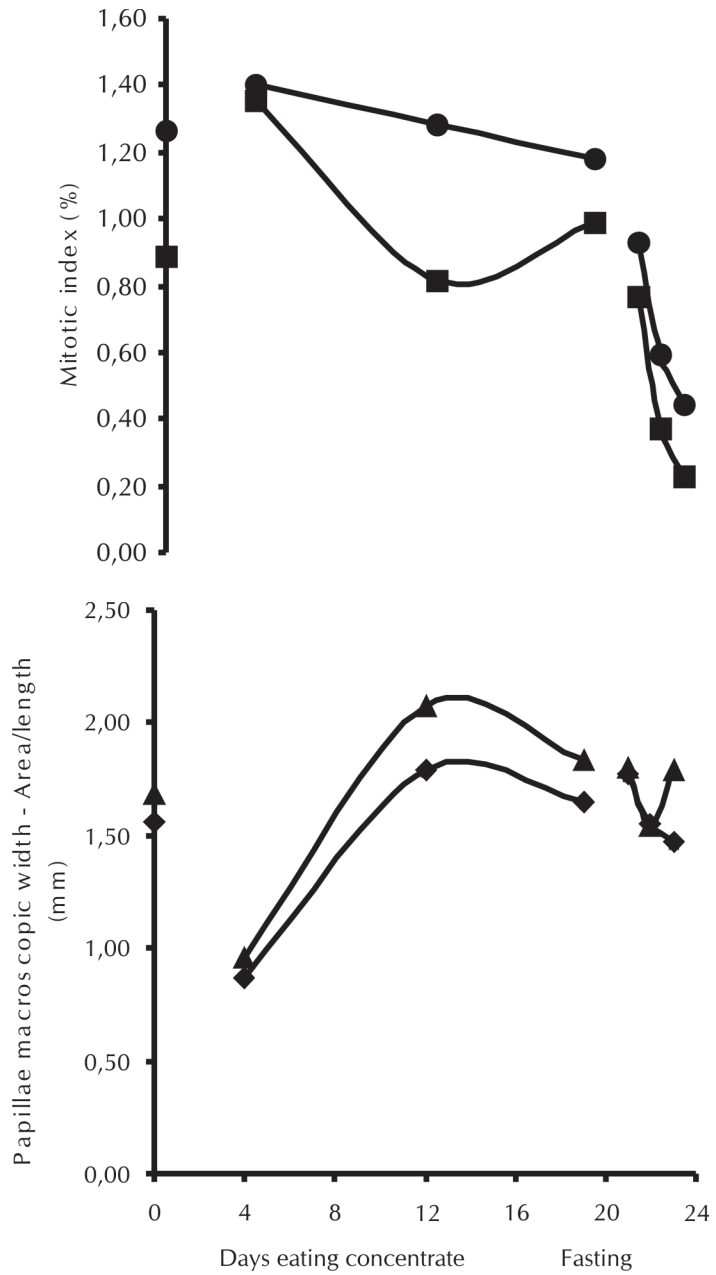
Figure 2 – Mitotic index of the ruminal ephitelium basal layer and ruminal papillae macroscopic width of non-lactating and non-pregnant cows fed with concentrate 1 (● ▲) or 4 (■ ♦) times a day and Coastal bermudagrass hay for 19 days followed by a 72 hours fasting period in experiment 1. Means adjusted for the same variable value at the end of the standardization period. (● ■) *P=0,03* for feed frequency effect; *P<0,001* for collect day effect; *P=0,49* for feed frequency and day interection. (▲ ♦) *P=0,55* for feed frequency effect; *P=0,07* for collect day effect; *P=0,65* for feed frequency and day interaction

fatty acids, especially butyrate, and insulin are probably stimulators of ruminal epithelium cellular proliferation[11,19]. Although there is no evidence showing that a ruminal epithelium, which presents a high mitotic index, also presents high metabolic cell activity and high VFA absorption capacity. In our study, the mitotic index was the best indicator of a morphological variable among all evaluated variables. The mitotic index can be useful in studies on nutritional induction of VFA absorption capacity by the rumen wall. Lower frequency of concentrate feeding resulted in a higher mitotic index in the basal layer of the ruminal epithelium (Table 1). Higher cellular division, however, was not associated to a papillae macroscopic or microscopic dimension increase. A concentrate feeding frequency effect on papillar size which is correlated to higher absorption capacity of ruminal epithelium[6,8] was not detected. Mitotic index has shown an acute response to changes in feeding plans[12,20]. Cellular proliferation, however, may not be the unique determinant factor of short term papilar size. Higher blood flow as a result of a larger amount of feed in the rumen and higher absorption necessity could result in papilar growth. Acute changes in rumen wall absorption capacity could not be associated only to papilar size[14]. In experiment 1, the mitotic index showed a higher value on day 4 of the comparison period, but it was not associated to larger papillar size (Figure 2). As the epithelium dimension is a result of the process of cellular synthesis and deletion[21], the acute effect of the positive changes in the feeding plan was not sufficient to induce cellular proliferation that could be reflected in larger papillae size[20]. A negative fasting effect on mitotic index and papillar size, however, was acute and unidirectional (Figure 2). The reduction of dry matter feed intake observed in dairy cows around calving[13], could be contrary to possible gains in terms of papillar morphology reached by higher concentrate feeding before calving. More information is needed concerning the relationship among

cellular proliferation rates, papillar size, and ruminal epithelium absorption capacity aiming at good ruminal acidosis control in dairy cows. The mitotic index in experiment 1 was higher 4 days after the beginning of the treatment, but went down on days 12 and 19 (Figure 2) to similar values observed at the end of the standardization period. The concentrate introduction in the diet probably caused higher VFA production demanding a higher ruminal epithelium absorption capacity. In the beginning cells presented a higher mitotic division rate to reach epithelial mass needed by the new VFA pattern. When this epithelial mass was reached, the cellular division rate returned to initial values needed for normal epithelium turnover. This variation profile on the mitotic index of the rumen papilla basal layer was observed in ovines submitted to VFA intraruminal infusion[12,22] or changes in feeding from roughage to concentrates[20]. The mitotic index values (Figure 2) followed a variation pattern negatively associated to ruminal pH and positively associated to plasmatic insulin value (Figure 3). Correlation between ruminal pH and VFA concentration in the rumen is negative and high[23]. VFA, especially butyrate, and insulin are probably stimulators of ruminal epithelium cellular proliferation[11,18]. Butyrate is a strong stimulator of insulin secretion[24,25] and was proportionally high in ruminal microorganism cultures with low pH values[26]. A decline in the relative proportion of acetate and a increase in the propionate proportion was detected in the sheep rumen that had a diet changed from roughage to concentrate[20]. Volatile fatty acids apparently induced systemic insulin secretion, which could have had an indirect stimulator action of the ruminal epithelium mitotic index. Elucidation of the direct and indirect effect of the VFA on ruminal epithelium cellular proliferation and differentiation in dairy cows could lead to a better understanding of the determinant mechanisms of ruminal absorption capacity and of ruminal acidosis occurrence in these animals. The higher mitotic index observed in animals fed once
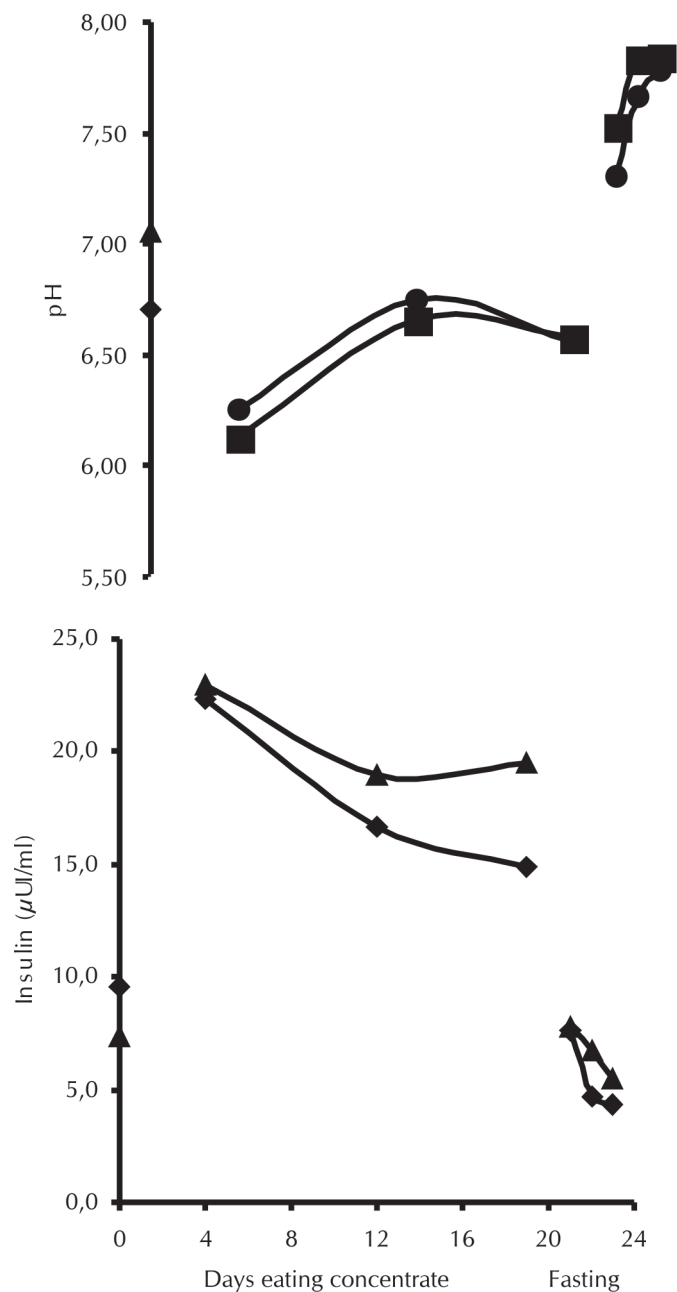
Figure 3 – Ruminal pH and plasmatic insulin of non-lactating and non-pregnant cows fed with concentrate 1 (● ▲) or 4 (■ ◆) times a day and Coastal bermudagrass hay for 19 days followed by a 72 hours fasting period in the experiment 1. Means adjusted for the same variable value at the end of the standardization period. (● ■) $P = 0,84$ for feed frequency effect; $P < 0,001$ for collect day effect; $P = 0,80$ for feed frequency and day interaction. (▲ ◆) $P = 0,54$ for feed frequency effect; $P < 0,001$ for collect day effect; $P = 0,86$ for feed frequency and day interaction

a day (Table 2 and Figure 2) was associated to variation in the ruminal pH values and plasmatic insulin over time (Figure 4), showing a non steady state condition in these animals. Relatively high pH at time zero of feeding (Figure 4) probably show a low VFA concentration because almost 24 hours had passed from the later feeding. The slight increasing of pH in the first 90 minutes after feeding could have occurred because during chewing action there is a high saliva production which has a buffering effect on ruminal pH. A decline in plasmatic insulin observed at this time (Figure 4) could suggest lower VFA absorption rates because the pH was relatively higher[27]. A strong linear decline of the pH associated with an inverse proportion of plasmatic insulin was observed at times after 90 minutes (Figure 4). This occurred probably because of the increase VFA concentration[23] in response to the fermentation of a larger amount of rapidly fermentable carbohydrate ingested only once. Concentrate feeding divided in 4 times a day was apparently efficient to maintain constant ruminal fermentation conditions, associated to constant plasmatic insulin levels and lower ruminal epithelium cellular division (Table 2).

Feeding concentrate frequency in cows and 72 hour fasting did not have an effect on the apoptotic index (Table 2 and 3). Tamate and Feel[21] reported that there was an apparent increase in the amount of apoptose in the ruminal epithelium basal layer cells in ovines submitted to fasting. These authors, however, did not measure apoptotic index. Data from these 2 experiments do not support the apoptotic index utilization, obtained in microscopic fragments stained with hematoxylin and eosin, as a good response variable to change in feeding plans.

There was no observed effect of the nutritional plan on papillae width, connective tissue width and number of epithelial pegs. These microscopic variables evaluated are indirect indicators of cellular mass possibly associated to rumen wall VFA absorption capacity[7,8]. The lack of response to these morphological markers could have occurred because probably the treatment application period was not long enough to induce changes in the papillar mass. Dirksen et al.[6] observed maximum papillar development 4 to 5 weeks after calving in Holstein cows, which were eating, concentrate for 6 to 7 weeks. In sheep the total epithelium turnover time was 16.5 days with roughage diet, 4.3 days at transition from roughage to concentrates and 10.9 days after several months eating concentrate[20]. Another plausible explanation for the lack of change in the microscopic parameter evaluated is the site where papillae were collected. Beharka[28] found that papillae from the cranial sac were less morphologically influenced by dietary variation than papillae from the ventral and dorsal sac.

Fasting induced during 72 hours in experiment 1 and 2 could not induce changes in papillae size, established by analysis of haematoxylin eosin stained glasses, showing there was no morphological response to accentuated nutritional changes especially in energy. However, when the area/length relation was established macroscopically from intact papillae we could observe an acute decrease on value at fasting period. (P<0,001 for day effect). Correlation between macroscopic width established by area/length relation and microscopic width determination in haematoxylin eosin stained glasses was 0,51. Although there exists a positive correlation between this variable, measurements in intact papillae seem to represent ruminal papilla area better than measurements in histologically prepared papillae.

Conclusions

Although low concentrate feeding frequency did not induce macroscopic differences in rumen papillae the response in ruminal pH, plasmatic insulin, and mitotic index of ruminal epithelium suggest that low concentrate feeding frequency, some weeks before calving, may be a management strategy able to induce papillar growth and potentially
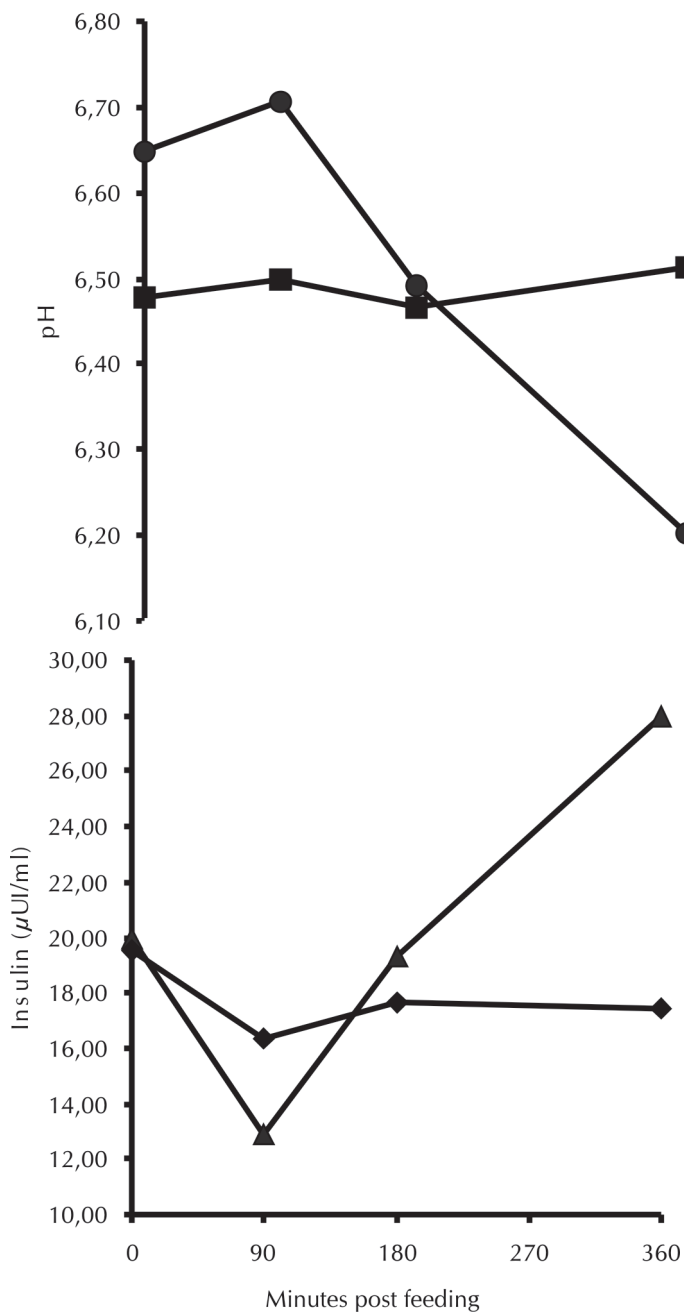
Figure 4 – Ruminal pH and plasmatic insulin of non-lactating and non-pregnant cows fed with concentrate 1 (● ▲) or 4 (■ ◆) times a day and Coastal bermudagrass hay for 19 days in the experiment 1. Mean values of each time adjusted for the same variable value at the end of the standardization period. (● ■) *P < 0,02* for feed frequency and time interaction; *P = 0,83* for feed frequency and day interaction. (▲ ◆)*P < 0,001* for feed frequency and time interaction; *P = 0,79* for feed frequency and day interaction

able to reduce acidosis incidence in the post-calving period in dairy cows. Accentuated fasting, however, can have detrimental effect on ruminal papillae morphology. Among morphologic variables evaluated, the mitotic index was the only one that presented a significant response to concentrate feeding frequency and to fasting in bovines and ovines. The mitotic index seems to be a good marker for quantification of the ruminal epithelium morphologic response to changes in feeding plans.

Efeito do padrão alimentar sobre a morfologia da parede ruminal de vacas e carneiros

Resumo

A manipulação nutricional da capacidade de absorção de ácidos graxos voláteis pela parede do rúmen pode ser uma estratégia para controlar acidose em vacas leiteiras. Objetivando induzir variação morfológica da parede do rúmen através da dieta e estabelecer marcadores morfológicos eficientes para epitélio ruminal, dois experimentos foram relizados. No experimento um, sete vacas com cânula ruminal foram alimentadas com concentrado uma ou quatro vezes ao dia por 19 dias seguidos por 72 horas de jejum. Papilas ruminais foram coletadas nos dias zero, quatro, 12 e 19 do período de tratamento e 24, 48 e 72 horas após o início do período de jejum. Baixa freqüência de alimentação concentrada foi associada a um aumento de insulina plasmática através do tempo (*P=0,02*) e a um maior (*P=0,03*) índice mitótico (IM), mas não afetou outros parâmetros morfológicos. No experimento dois, foram realizados dois ensaios não-simultâneos com três ovinos canulados no rúmen, os quais foram submetidos abruptamente a 72 horas de jejum. Papilas ruminais foram coletadas no final do período de alimentação e no final do jejum. O IM foi mais alto no período de alimentação do que no período de jejum (*P<0,01*), mas outros parâmetros morfológicos não foram capazes de responder à variação nutricional. Entres os marcadores morfológicos estudados o IM parece ser a melhor variável para avaliação da resposta morfológica do epitélio ao plano alimentar. A freqüência de alimentação concentrada pode ser usada para regular a morfologia das papilas ruminais.

Palavras-chave:
Rúmen.
Papila.
Índice Mitótico.
Insulina.
Gado Leiteiro.

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