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Original article

A protein-free diet alters small intestinal architecture and digestive enzyme activities in rats

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Abstract – The consequences of feeding a protein-free (PF) diet, as compared to casein, on gut characteristics were studied in slightly energy-restricted rats fed similar amounts of feed for 10 d. The weight and pH of fresh digesta in the stomach were lower ($P = 0.045$ and $P = 0.016$). However, the weight of fresh digesta in the other segments and gut tissue weight were not significantly affected by the diet ($P > 0.05$). Small intestinal crypt depth, width and area were reduced by 13, 23 and 37%, respectively ($P = 0.011$, $P = 0.004$ and $P = 0.001$), and villus width tended to be smaller ($P = 0.057$), with the PF diet. Villus height to crypt depth ratio was also lower with the PF diet in the duodenum and ileum, respectively ($P < 0.05$). Finally, the specific activities of alkaline phosphatase and aminopeptidase N were reduced by 36 to 38% at different sites of the small intestine in the rats fed the PF diet ($P < 0.05$). In conclusion, chronic consumption of a protein-free diet altered the intestinal villus-crypt architecture and enzyme activities in rats.

rat / protein-free diet / intestine / villus-crypt architecture / enzyme

1. INTRODUCTION

Feeding a protein-free (PF) diet to animals is common practice in digestion experiments in order to determine the so-called basal endogenous protein losses and, therefore, the true digestibility of dietary protein [1, 2]. This has been questioned since it is non physiological [3] and it affects protein synthesis rate of the whole body and the amount of protein entering the gut [4]. In baby calves, small intestinal villus-crypt architecture and digestive enzyme activities of the mucosa were drastically reduced with a PF diet [5]. However, literature data in rats are scarce, despite the numerous studies on gut endogenous protein losses in this animal species [6–8]. Chronic malnutrition and starvation in rats impair the gross anatomy of the gut, intestinal epithelial crypt cell proliferation (CCPR) and digestive enzyme activities [9–11].

Owing to the lack of specific data, we investigated the impact of a PF diet on gut weight characteristics and small intestinal morphology and mucosal enzyme activities

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Table I. Composition of the experimental diets.

	Diet[1]	
	Casein	PF
Ingredients (g·kg^{-1})		
Casein[2]	118	0
Starch	582	700
Others[3]	100	100
Vitamin-trace elements[4]	10	10
Analysis (g or kcal·kg^{-1} DM)		
Protein (N × 6.25)	109	9
Ether extract	65	62
Ash	71	64
NDF	63	68
Energy	3909	3967

[1] PF: protein-free diet.
[2] Casein was supplemented with 30 g DL-methionine per kg dry matter.
[3] Others (g·kg^{-1}) : sucrose 100, ground rice hulls 80, vegetable oil 60, sodium chloride 10, calcium carbonate 15 and calcium phosphate 25.
[4] Mineral and vitamin mixture supplied per kg of diet: 25000 IU vitamin A; 5000 IU vitamin D3; 20 IU vitamin E; 6 mg vitamin K; 10 mg vitamin B2; 35 mg calcium pantothenate; 75 mg niacin; 2.5 mg vitamin B6; 0.05 mg vitamin B12; 0.05 mg biotin; 200 mg choline; 150 mg Mn; 500 mg Zn; 40 mg Cu, 200 mg Fe; 2 mg I; 0.5 mg Se, 1 mg Co.

in rats. They were fed similar amounts of feed because dry matter intake influences intestinal architecture in rats [12, 13] and increases endogenous protein excretion in pigs [14].

2. MATERIALS AND METHODS

2.1. Animals and diets

The experiments were conducted in agreement with the guidelines of the National University of Colombia for the care and use of laboratory animals. Ten female Wistar rats (donated by the Zoo of Cali) were blocked into pairs and randomly allocated to the casein or PF treatment. Their initial body weights were 253 ± (SEM) 15 and 256 ± 19 g, respectively. The rats were kept in individual metabolic cages (Tecniplast 150-300; Buguggiate, Italy) for the whole experimental period. The control diet contained 118 g casein·kg^{-1} as the sole protein source, i.e. 100 g protein·kg^{-1} as recommended by FAO/WHO for the determination of the nutritional value of dietary proteins in rats [15]. Casein was enriched in methionine (30 g·kg^{-1} DM) and was substituted with starch in the PF diet (Tab. I). Casein was from bovine milk (reference C7978, Sigma, St. Louis, MO, USA).

2.2. Experimental conditions

Daily feed allowance was set at 10 g in order to limit feed refusals in this study. In fact, the whole experiment comprised other diets with increasing levels of phaseolin, the bean storage globulin of *Phaseolus vulgaris*, and it was aimed at determining the apparent and true digestibility of this protein (Montoya C.A. et al, 2004, unpublished). Since rats offered diets with high levels of phaseolin made some feed refusals in a pre-trial, and given the fact that the level of feed intake has an impact on small intestinal architecture and enzyme activities, we decided to apply a minimal feed restriction to the rats. As a consequence, they were slightly energy-restricted (89% of maintenance requirements). After a period of 10 d, the rats were fasted for the rest of the day. The following day, they received a single meal, 3 h before being sacrificed by asphyxia with chloroform followed by exsanguination. This post-prandial time has been considered as optimal for ileal digestibility studies in rats [6, 8]. The rats were weighed and their abdomen was opened immediately. The digestive tract was removed, weighed and positioned on an ice-containing tray covered with a glass square. Segments including the stomach, caecum and colon were isolated and weighed filled and empty. The pH of the contents of the stomach and the caecum was also measured. After being unrolled and the length measured, the small intestine was divided into two halves by length, emptied and the tissues and contents were weighed. This was

also done with the whole colon. Samples (1.5 cm in length) of the small intestine were taken for histology at 10 cm distal from the pylorus (duodenum), in the middle of the small intestine (jejunum) and 10 cm before the ileo-caecal junction (ileum). Each fragment was cut longitudinally and washed with distilled water before being fixed in buffered formalin and kept at 4 ºC until morphology analysis [5]. Other samples of 3 cm in length were collected at the same sites of the small intestine and immediately frozen in liquid nitrogen before enzyme activity determination.

2.3. Analyses

The diets were analysed for ash, nitrogen, ether extract and neutral detergent fibre using conventional analytical methods.

Small intestinal morphology was assessed using microdissection [16]. Villus and crypt length, width and surface area were measured using image analysis [5]. Mean values of these parameters were determined for 15 individual villi and 10 crypts from each specimen.

All the reagents used for the determination of intestinal enzyme activities were from Sigma. The frozen small intestinal samples were thawed on ice and homogenised in ice-cold saline (NaCl 0.9%) and refrozen at –40 °C until analysis [5]. The specific activities of alkaline phosphatase (EC 3.1.3.1) and aminopeptidase N (EC 3.4.11.2.) was determined according to [17] and [18], respectively. These enzymes were retained because the former is considered a biomarker of intestinal maturation and the latter was found to vary similarly to other peptidases (aminopeptidase A and dipeptidylpeptidase IV) in young calves fed milk replacers with different protein contents [5]. Protein was measured [19] and specific enzyme activities were calculated.

2.4. Statistical analyses

An analysis of variance of the data was conducted using the GLM procedure of SAS [20] in order to test the effect of the diet and, when relevant, the effects of the intestinal site and diet by site interaction. This was carried out for digesta pH, small intestinal morphology and enzyme activities according to a split-plot design, considering block error as a residual variance to test the effect of diet and the residual error to test the effects of intestinal site and diet by site interaction. When the F-value of the analysis of variance was significant ($P < 0.05$), the means were compared by the Least Squares Difference procedure of SAS. Data are presented as means and SEM.

3. RESULTS

3.1. Technical data

The rats consumed similar amounts of casein and PF diets daily (9.35 ± 0.37 and 9.46 ± 0.17 g, $P = 0.64$). This was also the case for the last meal before sacrifice where they consumed virtually all their daily allowance. The final body weight of rats did not significantly differ between the diets (238 ± 13 and 224 ± 18 g, $P = 0.21$).

3.2. Gut tissue and digesta characteristics

The dietary treatment did not have a significant effect ($P > 0.05$) on either the tissue weight of the whole gut and its segments relative to bodyweight nor the lengths of the small intestine and colon (data not shown).

The PF rats presented a 72% reduction in the weight of fresh digesta in the stomach ($P = 0.045$) (Tab. II). The weight of fresh contents in the other segments was not affected by the diet ($P > 0.10$). A significant diet by site interaction was observed for the pH of the gut contents ($P = 0.001$). Gastric pH was significantly lower in the PF rats than in the casein controls (5.33 ± 0.20 and 3.70 ± 0.34, $P < 0.05$) while caecal pH remained unaffected by the dietary treatment (7.51 ± 0.05 and 7.69 ± 0.08, $P > 0.05$).

Table II. Influence of the diet on the weight of fresh digesta in the gut and its segments in rats fed a casein or a protein-free diet (g·100 g^{-1} bodyweight) (means and SEM, $n = 5$ per treatment).

	Diet[1]		*P*
	Casein	PF	
Stomach	2.20 (0.18)	0.62 (0.24)	0.045
Small intestine	1.65 (0.16)	1.51 (0.10)	0.31
Caecum	0.61 (0.06)	0.82 (0.12)	0.11
Colon	0.41 (0.12)	0.27 (0.08)	0.24

[1] PF: protein-free diet.

Digesta pH was also higher in the caecum as compared to the stomach ($P < 0.0001$).

3.3. Small intestinal architecture

No significant diet by site interaction was observed, except for intestinal villus height to crypt depth ratio ($P = 0.038$) (Tab. III). Villus height and area were not significantly influenced by the diet ($P = 0.22$) but villus width tended to be reduced ($P = 0.057$) in the PF rats. Crypt depth, width and area were higher in the PF rats ($P = 0.011$, $P = 0.004$ and $P = 0.001$, respectively). Small intestinal villus height to crypt depth ratio was lower in the duodenum of the PF rats as compared to the casein controls ($P < 0.05$). For all the variables studied, except villus and crypt width, the effect of intestinal site was significant ($P = 0.037$ to $P = 0.001$), with usually a decreasing proximo-distal gradient.

3.4. Protein content and enzyme activities of the small intestinal mucosa

The diet by site interaction and the diet effects for the protein content of the small intestinal mucosa were not significant ($P > 0.05$) (data not shown). The diet by site interaction was significant for the specific activities of alkaline phosphatase ($P = 0.0006$) and aminopeptidase N ($P = 0.027$) (Tab. IV). Alkaline phosphatase activity was 36% lower in the duodenum of the PF rats ($P < 0.05$), with no significant differences in the jejunum and ileum ($P > 0.05$). Aminopeptidase N activity was 38% lower in the duodenum and ileum, but not in the jejunum, of the PF rats ($P < 0.05$).

4. DISCUSSION

The major findings of the present study are deeper crypt architecture and reduced mucosal enzyme activities of the small intestine, with little effect on the villi, following chronic consumption of a protein-free diet in rats slightly restricted in energy.

Feeding a protein-free diet has been shown to have adverse effects on the body and various organs. It decreases the rate of body protein synthesis [4] and influences body composition [21]. At the gut level, it may reduce the levels of gastric and pancreatic enzyme secretion and increase the breakdown and re-utilisation of secreted enzymes [22, 23]. The absolute amounts of amino acids transported across the intestine are decreased but the absorptive capacity (per unit of intestinal DNA) is increased [24]. In the liver, the protein synthesis is decreased [4, 25] while the activity of gamma-glutamyl transpeptidase (EC 2.3.2.2.) is drastically increased [26]. An interesting transcriptomic study recently revealed that 281 genes were up- or down-regulated in the liver after feeding a protein-free diet to rats [27]. Two thirds of these genes correspond to genes already identified in receptor and signal transduction, transport and binding of proteins, amino acid metabolism

Table III. Influence of the diet on small intestinal morphometry in rats fed a casein or a protein-free diet (means and SEM, $n = 5$ per treatment).

Variable	Site[3]	Diet		P[2]		
		Casein	PF	Diet	Site	Diet × Site
Villus height (µm)				0.22	0.001	0.13
	D^x	492 (29)	418(22)			
	J^y	404 (12)	410 (8)			
	I^z	244 (16)	215 (18)			
Villus width (µm)				0.057	0.084	0.58
	D	391 (22)	345 (12)			
	J	350 (25)	328 (22)			
	I	292 (17)	280 (21)			
Villus area ($\mu m^2 \times 10^3$)				0.22	0.001	0.21
	D^x	181 (19)	142 (13)			
	J^y	135 (12)	129 (11)			
	I^z	68 (5)	62 (8)			
Crypt depth (µm)				0.011	0.016	0.31
	D^x	164 (11)	200 (9)			
	J^y	154 (7)	181 (6)			
	I^z	150 (5)	161 (7)			
Crypt width (µm)				0.004	0.19	0.20
	D	40 (4.5)	45 (2.5)			
	J	30 (1.8)	45 (1.6)			
	I	35 (2.2)	47 (2.0)			
Crypt area ($\mu m^2 \times 10^3$)				0.001	0.037	0.30
	D^x	5.0 (0.25)	8.2 (0.36)			
	J^y	4.0 (0.35)	7.1 (0.33)			
	I^z	4.5 (0.37)	6.4 (0.47)			
Villus height to crypt depth ratio				0.021	0.001	0.038
	D	3.1^a (0.24)	2.1^{bcd} (0.12)			
	J	2.7^{ab} (0.14)	2.3^{abc} (0.15)			
	I	1.6^{cd} (0.08)	1.3^d (0.06)			

[1] PF protein-free diet.
[2] Means with different superscripts for a given variable (a, b, c, d) or site (x, y, z) differ ($P < 0.05$).
[3] D: duodenum, J: jejunum, I: ileum.

Table IV. Influence of the diet on small intestinal enzyme activities in rats fed a casein or a protein-free diet (means and SEM, $n = 5$ per treatment).

Variable	Site[3]	Diet		P[2]		
		Casein	PF	Diet	Site	Diet × Site
Alkaline phosphatase[4]				0.027	0.001	0.001
	D	411^{a} (26)	264^{b} (24)			
	J	68^{c} (13)	81^{c} (19)			
	I	44^{c} (17)	41^{c} (12)			
Aminopeptidase N[5]				0.013	0.001	0.027
	D	38^{ab} (6)	24^{c} (4)			
	J	51^{ab} (2)	48^{ab} (4)			
	I	55^{a} (5)	34^{bc} (5)			

[1] PF protein-free diet.
[2] Means with different superscripts for a given variable (a, b, c) differ ($P < 0.05$).
[3] D: duodenum, J: jejunum, I: ileum.
[4] nmol substrate degraded/mg tissue protein/h.
[5] μmol substrate degraded/mg tissue protein/h.

and gene expression control, etc. One third of the genes were unassigned to particular molecules or functions, suggesting the consequences of protein deprivation to be much broader than anticipated. The effects of a protein-free diet are also reflected in the plasma. The levels and/or molar ratios of all indispensable amino acids and plasma proteins, including albumin, are decreased while those of several gluconeogenetic amino acids, especially glycine and alanine, and gamma glutamyl transpeptidase activity are all increased [26, 28]. Finally, protein synthesis and the levels of most amino acids were also decreased in the brain of rats fed a protein-free diet [29].

Here, rats consuming a PF diet had a lower gastric pH and accelerated emptying rate, probably due to the absence of casein and clotting in this compartment. Intestinal CCPR is under hormonal control through gastrin action [30–32]. Whether the crypt morphology changes observed here were influenced by changes in plasma gastrin levels is not known because we did not measure it in the present study.

Villus height was little affected by the dietary treatment here. This is in sharp contrast with our previous observations with preruminant calves fed milk replacers varying in protein content [5]. Villus height and width and crypt width and area were reduced by 20 to 30% in various parts of the small intestine with the PF diet. Milk replacers for calves contain high levels of lactose and fat, combined with low levels of starch and no fibre. These highly digestible diets, when they are protein free, are most probably completely digested in the proximal small intestine [5], with a resulting median to distal villus-crypt atrophy, as seen in models of total parenteral nutrition [33, 34]. Here, the experimental diets contained fibre and starch. This might have provided the minimal energy and load necessary for restoring intestinal atrophy observed in rats on total parenteral nutrition [34]. Studies with neonatal pigs have also shown that the minimal enteral nutrient intake necessary to increase mucosal mass is close to 40% of total nutrient intake [35].

The present enzyme activity results are in good agreement with published data showing reduced specific activities of alkaline phosphatase and aminopeptidase N in rats [9, 13, 36] and calves [5] fed diets low in protein. An influence of the amount of

starch, which was 20% higher in the PF diet, on enzyme activities cannot be excluded. However, increasing the protein level in a diet is often achieved at the expense of starch, thus leading to confounded effects. For example, diets with low starch and high casein contents result in higher specific activity of leucine-aminopeptidase than diets with high starch and low casein contents [37]. Intestinal alkaline phosphatase activity (but not that of maltase) has been found to be higher in rats fed a starch, as compared to sucrose, diet [38]. Here, the lack of difference in caecal pH between treatments only suggests that the differences in diet, and therefore starch composition did not significantly influence this factor.

In conclusion, feeding a PF diet to rats slightly restricted in energy affected gastric emptying and small intestinal mucosal architecture and enzyme activities. Our data support the view that dietary protein is stimulatory to intestinal mucosa [39] and endogenous protein secretion [7, 8].

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