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EFFECT OF SODIUM BUTYRATE ON THE SMALL INTESTINE DEVELOPMENT IN NEONATAL PIGLETS FEED BY ARTIFICIAL SOW

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Feeding of neonates with artificial milk formulas delays the maturation of the gastrointestinal mucosa. Na-butyrate has a complex trophic effect on the gastrointestinal epithelium in adults. The present study aimed to determine the effect of milk formula supplementation with Na-butyrate on the gut mucosa in neonatal piglets. Sixteen 3 day old piglets were randomly divided into two groups: control (C, n = 8), and Na-butyrate (B, n = 8). Animals were feed for 7 days with artificial milk formula alone (C) or supplemented with Na-butyrate (B). At the 10th day of life the piglets were sacrificed and whole thickness samples of the upper gut were taken for analyses. Administration of Na-butyrate led to significant increase in daily body weight gain as compared to control. In the duodenum, the villi length and mucosa thickness were reduced, however, in the distal jejunum and ileum, the crypt depth, villi length and mucosa thickness were increased in Na-butyrate supplemented piglets as compared to control. Supplementation with Na-butyrate did not affect the intestinal brush border enzyme activities but increased plasma pancreatic polypeptide and cholecystokinin concentrations. These results suggest that supplementation with Na-butyrate may enhance the development of jejunal and ileal mucosa in formula-fed piglets.

K e y   w o r d s : *artificial milk formula, intestinal villi, lactase, gut maturation, pig*

INTRODUCTION

Butyric acid is one of the short-chain fatty acid (SCFA) that is the C2-5 organic fatty acid. It is a main end-product of anaerobic bacterial fermentation of carbohydrates in the rumen of forestomach animal species and in the colon

of omnivorous species involving humans. In the experimental trials, sodium (Na-) butyrate is often used instead of butyric acid since it is solid, stabile and much less odorous. In the large intestine, Na-butyrate is rapidly absorbed to provide energy to the epithelial cells (1 - 3) and promote sodium and water absorption (4). Na-butyrate affects epithelial cell growth and differentiation, and increases the proliferation index in the intestinal crypts (5), and thereby reveals trophic effect on the gut mucosa. Moreover, it reveals anti-inflammatory effect on gastric and intestinal mucosal cells in adult rats (6). In contrast, in the colon cancer cell lines *in vitro*, Na-butyrate was revealed as a powerful inhibitor of growth and inducer of phenotype differentiation and apoptosis affecting a large variety of intracellular proteins (7, 8). Therefore Na-butyrate is considered to exert beneficial effects in reducing risk factors involved in the etiology of colon cancer and adenoma development. A term "butyrate paradox" was born to emphasize opposite effects of butyrate on the normal and neoplastic cells at the level of proliferation, differentiation, and gene expression (9).

Galfi and Bokori (10) were the first to show positive influence of Na-butyrate on the body weight gain, feed utilization and composition of intestinal microflora in growing pigs. They also indicated some trophic effects on the intestinal epithelium, like increase in the villi length and crypt depth. However, the role of Na-butyrate on intestine development in the neonatal pigs has not been investigated so far. Our recent studies showed, that artificial milk formulas provide much poorer stimulation to the developing intestine as compared with sow milk (11, 12). We hypothesise, that supplementation of milk formula with Na-butyrate may speed up growth and maturation of the gastrointestinal tract in newborn formula-fed animals. The present study aimed to determine the effect of milk formula supplementation with Na-butyrate on the structure of the stomach and small intestine mucosa, intestinal brush border enzyme activities, and plasma gastrin, cholecystokinin (CCK), and pancreatic polypeptide (PP) in neonatal piglets feed by "artificial sow".

MATERIALS AND METHODS

Animal experiments

The Local Ethics Committee approved the study protocol. Total 16 male crossbred neonatal piglets (pietrain x polish landrace) from 8 litters were used. Three day old piglets were taken from their sows, transported to the laboratory and placed in the artificial sow system providing *optimum* temperature, humidity and light (12/12 h dark/light cycle). Animals were divided into two groups (n=8), control (C) and experimental (B). Adaptation period was the first 10 h, when all piglets were kept in one box of artificial sow system to learn artificial feeding. After the adaptation, the piglets were placed into the individual cages provided social contact with each other. The animals were feed every 75 min (30 to 34 ml milk formula) by artificial sow (model Research Center-Foulum, Pig's oline, Boss' Produkter a/s Denmark). Pigs were feed for 7 days with artificial milk

formula for piglets Lakti R (Trouw Nutrition, Polfarm) consisting of 20 % protein, 17 % lipid, and 17,7 MJ metabolic energy. Experimental group received milk formula supplemented with 0,3 g sodium butyrate (Merck, Schuchardt, Germany) per 100 g dry matter. After 7 d the piglets were sacrificed by thiopental (Thiopental, Biochemie, Austria) overdosing, and the entire gastrointestinal tract was removed for morphometry analysis and tissue sampling. Liver, stomach, pancreas and small intestine were weighed. Whole-thickness samples of the duodenum, proximal (25 %), middle (50%) and distal (75%) part of jejunum, and ileum were fixed in Bouin solution for histological analysis. The corresponding 15-cm long intestine segments were gently scraped to collect the mucosa for enzyme activity analysis. Ten ml blood samples were harvested from the subclavian vein for gastrin, CCK and PP analyses just before the animals were sacrificed. Blood samples were centrifuged at 4000 rpm (4° C) for 15 min, and plasma was harvested and stored frozen (-20° C) until analysis.

Mucosa histology

Serial histological sections of 5-µm thickness were cut and stained with hematoxylin and eosin for morphometry analysis under a light microscope. Morphometry analysis involved measurements in the stomach (thickness of tunica mucosa and muscularis), and small intestine (depth of crypts, length of villi, thickness of tunica mucosa and muscularis) in 5 to 8 slides for each tissue sample. In each slide, thirty well-oriented villi and crypts lying outside the Peyer's patches were measured at a low magnification with a Nikon optical binocular microscope (using Lucia G v.4.60 software) coupled via digital camera to a computer (12).

Brush border enzyme activity

Brush border enzyme activity was measured in the mucosa scrapings from the proximal, middle and distal jejunum. The mucosa was scraped off with a microscope slide, and deep-frozen (-80° C). After thawing, the intestinal mucosa was homogenized in cold distilled water (1g intestinal mucosa/5 ml distilled water) and centrifuged for 5 min at 1000 g and 4° C. The protein content was determined using modified Lowry method (12) employing using bovine serum albumin as the standard. The activities of aminopeptidase A and N were assayed with L-glutamyl-p-nitroanilide and L-leucyl-p-nitroanilide as substrates, respectively (13), and that of dipeptidase IV was assayed with glycyl-L-prolyl-p-nitroanilide (14). The resulting enzymatic units (IU) were expressed as µmol of p-nitroanilide released/min at 37° C. Lactase, maltase and sucrase were determined as described by Dahlquist (15) with minor modifications.

Gastrin, CCK and PP radioimmunoassay

Plasma concentrations of gastrin, CCK and PP were measured by radioimmunoassay using a double antibody technique. Concentration of gastrin was measured according to Chayvialle *et al.* (16), and PP according to Miazza *et al.* (17). CCK was analysed using commercially available kit (EURIA-CCK, Eurodiagnostica, Malmo, Sweden).

Statistical analysis

Data are expressed as their means ± standard errors of mean (SEM). The unpaired Student's t-test or nonparametric Mann-Whitney test were used to indicate the statistical differences between the groups (Graph Pad Software version 4.0, San Diego, CA, USA). In all statistical analysis $P<0.05$ was taken as the level of significance.

RESULTS

Body weight and morphometric measurements

The body weight gain is shown in *Table 1*. The initial body weight of piglets in the experimental group was significantly higher as compared to control group. The final body weight and the daily body weight gain were significantly higher in experimental animals as compared with control. Morphometric analysis did not show any significant differences in the length of small intestine segments between the C and B groups (*Table 2*).

Histometry analyses

In the stomach, the tunica mucosa was significantly thicker in Na-butyrate supplemented piglets as compared to control (B group: 449 ± 41 µm *vs*. C group: 337 ± 57 µm; $P \le 0.001$). Also the tunica muscularis was significantly thicker in Na-butyrate supplemented as compared to control animals (508 ± 103 µm *vs*. 361 ± 52 µm; $P \le 0.001$).

Supplementation of milk formula with Na-butyrate led to significant changes in the histometry parameters of the small intestine (*Table 3*). The crypt depth, in the proximal and distal jejunum and ileum was significantly higher, whereas in the mid jejunum it was significantly lower in Na-butyrate supplemented piglets as compared to control. There was also a tendency toward increase (not significant) in the duodenal crypts. The longer villi were observed in the proximal and distal jejunum and ileum of Na-butyrate supplemented piglets, and shorter in the duodenum as compared to controls. In the mid jejunum, there was a tendency toward an increase in the villi length, but not significant. In Na-butyrate

Table 1. Initial and final body weight (kg) at the 3rd and 10th day of life, respectively, and daily body weigh gain (kg/d) in control (C) and Na-butyrate supplemented (B) piglets.

Groups	Initial body weight (kg)	Final body weight (kg)	Mean daily body weight gain (kg/d)
C	1.72 ± 0.20	2.16 ± 0.14	0.06 ± 0.02
B	$2.15 \pm 0.19**$	$2.76 \pm 0.14***$	$0.09 \pm 0.02*$

Values are given as means $\pm$ SEM (n=8). Asterisks indicate statistical significance (unpaired Student's t-test or nonparametric Mann-Whitney test (* ≤ 0.05; ** ≤ 0.01; *** ≤ 0.001).

Table 2. Length of the entire small intestine and the duodenum, jejunum and ileum (cm) in control (C) and Na-butyrate supplemented (B) pig neonates.

Group	Small intestine (cm)	Duodenum (cm)	Jejunum (cm)	Ileum (cm)
C	535 ± 70	8.3 ± 1.3	518 ± 69	8.5 ± 1.7
B	523 ± 48	9.1 ± 1.7	505 ± 47	8.5 ± 1.6
P	*0.74*	*0.35*	*0.72*	*0.95*

Values are given as means $\pm$ SEM (n=8). Unpaired Student's t-test.

Table 3. Crypt depth, villous length and tunica mucosa and muscularis thickness (mm) in the duodenum, jejunum (25%, 50% and 75% of length from ligament of Treitz) and ileum of control (C) and Na-butyrate supplemented (B) neonatal piglets.

Intestine segment	Groups	Crypt depth (μm)	Villi length (μm)	Mucosa thickness (μm)	Muscularis thickness (μm)
Duodenum	C	132 ± 24	359 ± 31	525 ± 44	78 ± 13
	B	158 ± 51	330 ± 40**	461 ± 44***	96 ± 16***
25%	C	130 ± 35	323 ± 37	479 ± 30	61 ± 13
	B	142 ± 23***	378 ± 61**	526 ± 121	74 ± 11***
50%	C	141 ± 27	341 ± 33	495 ± 22	70 ± 8
	B	122 ± 14***	366 ± 55	536 ± 41***	62 ± 10***
75%	C	122 ± 30	344 ± 33	480 ± 74	70 ± 11
	B	132 ± 10***	400 ± 47***	550 ± 42***	71 ± 11
Ileum	C	116 ± 24	305 ± 42	453 ± 51	87 ± 10
	B	123 ± 14***	350 ± 62***	521 ± 56***	110 ± 26***

Values are given as means ± SEM (n=8). Asterisks indicate statistical significance (unpaired Student's t-test or nonparametric Mann-Whitney test (** ≤ 0.01; *** ≤ 0.001).

supplemented group the *tunica mucosa* was significantly thicker in the jejunum and ileum as compared to control. In contrast, the duodenal mucosa was significantly thinner. Measurements of the tunica muscularis showed significant increase in the duodenum, proximal jejunum and ileum of Na-butyrate supplemented as compared to control piglets.

Brush border enzyme activities

Brush border lactase, maltase and sucrase activities decreased along the jejunum in the both groups of piglets (*Table 4*). On the other hand, in aminopeptidase A and N and dipeptidase IV activities a tendency toward slight increase along the gut can be observed in C and B groups of piglets. In comparison with the control, Na-butyrate supplementation did not affect the brush border enzyme activity (*Table 4*).

Plasma gastrin, CCK and PP

Plasma gastrin concentration was not different between the two groups (*Table 5*). The concentration of plasma CCK was almost 4-fold higher in Na-butyrate supplemented piglets as compared to control. In contrast, plasma PP was 2.5-fold lower in the Na-butyrate supplemented group.

DISCUSSION

In the present study butyric acid was used in the form of its sodium salt since it could easily be mixed in the diet. Moreover, Na-butyrate unlike the commonly

Table 4. Brush border lactase, maltase, sucrase, aminopeptidase A (Amp. A), aminopeptidase N (Amp. N) and dipeptidase IV (Dpp. IV) activities (µmol/min per g of mucosa) in the proximal (25%), mid (50%) and distal (75%) jejunum of control (C) and Na-butyrate supplemented (B) pig neonates.

		C	B	*P*			C	B	*P*
Lactase	25%	3.83 ± 2.12	5.20 ± 4.10	*0.44*	Amp. A	25%	6.1 ± 2.3	4.2 ± 2.6	*0.27*
	50%	2.71 ± 1.48	2.01 ± 2.99	*0.21*		50%	6.2 ± 2.0	6.6 ± 3.8	*0.84*
	75%	0.66 ± 0.28	1.79 ± 1.28	*0.38*		75%	7.2 ± 1.9	7.6 ± 2.6	*0.80*
Maltase	25%	10.73 ±7.04	9.05 ± 7.60	*0.69*	Amp. N	25%	4.7 ± 1.4	3.0 ± 1.2	*0.08*
	50%	5.32 ± 3.32	6.51 ± 2.94	*0.58*		50%	4.7 ± 1.6	2.9 ± 1.5	*0.09*
	75%	2.60 ± 0.74	3.24 ± 1.38	*0.57*		75%	5.0 ± 2.6	4.5 ± 1.0	*0.80*
Sucrase	25%	5.69 ± 2.56	2.99 ± 2.05	*0.13*	Dpp. IV	25%	1.2 ± 0.4	1.1 ± 0.7	*0.90*
	50%	3.83 ± 3.30	1.63 ± 0.79	*0.63*		50%	1.3 ± 0.5	1.3 ± 1.0	*0.96*
	75%	0.53 ± 0.13	0.58 ± 0.30	*0.80*		75%	1.6 ± 0.5	2.2 ± 1.0	*0.50*

Values are given as means ± SEM (n=8). Unpaired Student's t-test or nonparametric Mann-Whitney test.

Table 5. Plasma gastrin, cholecystokinin (CCK) and pancreatic polypeptide (PP) radioimmunoactivities (pmol/l) in the control (C) and Na-butyrate supplemented (B) neonatal piglets.

Groups	Gastrin	CCK	PP
C	159.2 ± 13.3	2.3 ± 1.5	121.6 ± 22.9
B	134.6 ± 48.7	8.0 ± 4.2***	48.7 ± 13.9***

Values are given as means ± SEM (n=8). Asterisks indicate statistical significance (unpaired Student's t-test; *** $P \le 0.001$).

used organic acids does not decrease the pH of the diet (10) what is particularly important in the neonates. Thus, Na-butyrate effects are due to broad biological actions of the molecule rather than to its acidic character.

The final body weight was higher in Na-butyrate supplemented animals, but this result needs to be considered cautiously since the initial body weight gain was also higher. Higher initial body weight in the experimental group was a consequence of random allocation of piglets into the control and experimental groups. Nevertheless, the influence of Na-butyrate may be more reliably evaluated by the disproportion seen in the daily body weight gain between the

two experimental groups. Namely, the Na-butyrate supplemented piglets gained daily on the average 1.5-fold more than the control piglets. These results corroborate with those obtained in older pigs by Galfi and Bokori (10), and we can conclude that Na-butyrate may lead to increase in daily body weight gain in piglets.

Supplementation with Na-butyrate led to a number of trophic changes in the structure of the stomach and small intestine. An increase in the stomach *tunica mucosa* and *tunica muscularis* has not been reported yet for Na-butyrate, though present data suggest that further studies on the role in gastric mucosal cytoprotection and blood flow (18, 19) are necessary. Recently, Dembiński *et al* (20) reported that exogenous PP reduces gastric mucosa growth in the rat, thus we may speculate that the growth effects in the present piglets could be in a part attributed to a significant reduction in plasma PP (*Table 5*). Trophic effects on the pig intestine reported by Galfi and Bokori (10) were limited to the ileum and *caecum*. There were no data available on the remained segments of the small intestine. In our study, Na-butyrate revealed its trophic effect on the jejunal and ileal mucosa but not the duodenal mucosa, though it apparently increased the CCK release from the CCK-producing cells. The increased crypt depth normally coincide with increased mucosal secretion rather than with increased mitosis, thus to evaluate the proliferation rate, a mitotic index count is necessary. Salminen (5), reported that Na-butyrate significantly increase stem cell proliferation in the intestinal crypts. By increasing the length of the intestinal villi, Na-butyrate enlarges the absorptive surface of the gut in particular in the proximal and distal jejunum and ileum thereby favorably influencing the absorption of nutrients from the gut lumen. More molecular studies need to be done to verify this hypothesis. In contrast, no corresponding changes were observed in the duodenum, maybe because of quick chyme flow through this segment of the gut. The trophic changes in the proximal jejunum could be easily ascribed to Na-butyrate readily absorbed by the gut (1, 2). In organ culture of human colonic mucosa *in vitro*, butyrate was shown to directly stimulate the epithelial cell proliferation in the absence of circulating neural factors (21). However, how to explain the changes found in the distal jejunum and ileum? The mechanism(s) is not clear, but orally ingested Na-butyrate seems to indirectly rather then directly mediates the growth of the distal small intestine. Butyric acid when installed into the colon was shown to exert potent systemic trophic and mitotic effects on the unexposed epithelium of the ileum and jejunum (22-24). Moreover, SCFA infusions into the colon lumen stimulated growth of isolated, denervated jejunal loops in rats (25). This effect may be specific to butyric acid/butyrate, since infusions of other SCFAs were trophic to the colonic mucosa only when infused directly into the colon. Thus, the trophic effects of the other SCFAs on the colon are mediated locally (24). In our piglets, Na-butyrate showed trophic effect on smooth muscles in the duodenum, proximal jejunum and ileum suggesting that hypertrophy may involve not only the gut tunica mucosa. In consequence a small intestinal motility may be

enhanced, thereby, changing the conditions of *digesta* passage and their absorption. The overall changes in the gut in present piglets may lead to improved feed utilization resulting in increased body mass gain. Further studies on molecular event and mitotic and apoptotic indexes in the gut mucosa are warranted to strengthen this hypothesis.

Frankiel and co-workers (24) have shown that SCFA mixture (150 mmol L acetic, 30 mmol/L propionic, 90 mmol/L butyric acids) infusions into the rat isolated (but not denervated) cecum caused trophic effects in the jejunal mucosa. Their results suggest the participation of autonomic nerves in mediating the jejunotrophic effects of SCFAs. In their study, jejunal gastrin levels were also significantly increased suggesting that gastrin may be another mediator of SCFA-induced jejunotrophism. In our study, however, we did not observe any increase in plasma gastrin concentration. The differences between the two studies may be related to the differences in the composition and/or the site of administration. Previously, Biernat and co-workers (11, 26) have shown that endogenous CCK may play fine but significant role in regulating the small intestinal structure and function development in neonatal calves and pigs. Therefore, marked increase in plasma CCK observed in the present study may contribute to an enhanced development observed in the gut of Na-butyrate supplemented piglets. In contrast plasma PP was reduced what may suggest decline in neural vagal activity (27), a result being in opposition to that reported by Frankiel and co-workers (24) but favouring that recently described by Dembiński *et al* (20).

The activity of brush border enzymes showed no changes between the groups, and normal activity gradient along the jejunum. No literature data on the brush border enzyme activities are available for comparison. Based on our results we may conclude that in neonatal piglets Na-butyrate seem not to affect the enterocyte activity to synthesize the brush border enzymes (both sucrases and peptidases). It is known, however, that the activity of other brush border membrane enzyme such as alkaline phosphate is increased by butyrate in colon cancer cells (28). Butyrate is also a potent inhibitor of urokinase secretion in normal colon and Lim-1215 colon cancer cells (29). Feeding with milk formula delays the physiological changes in the pattern of gut brush border enzyme activities normally observed in suckling neonates (11, 12) and is considered not beneficial for the newborns. Thus, in milk formula fed piglets the decrease in lactase, maltase and sucrase activities, and increase in endopeptidase activities as compared to sow reared piglets were observed probably due to the absence of milieu of bioactive substances in the milk formula. Apparently, Na-butyrate added to milk formula could not replace these bioactive substances in regard to brush border enzyme activity regulation.

In conclusion, present data suggest that supplementation of milk formula with Na-butyrate contribute to enhanced development of the intestinal mucosa in neonatal piglets.

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