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Preliminary results on exogenous ghrelin administration *via* stomach tube influence on the crypt cell proliferation in the small intestine mucosa of neonatal piglets*

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ABSTRACT

The aim of this study was to investigate the exogenous ghrelin effect on proliferation in the small intestinal mucosa, body and organs weight of neonatal piglets. Four groups (n=6) of pig neonates were examined: sow reared (C7SR), fed with milk formula (C7), fed with milk formula + ghrelin administrated *via* stomach tube in the dose of: 7.5 μg/kg body weight (BW) (G7.5) and 15 μg/kg BW (G15). Milk formula feeding (C7) strongly decreased mitotic rate in all parts of jejunum as compared to C7SR. Ghrelin treatment (G7.5) had a pro mitotic effect on the middle part of the small intestine ($P<0.0001$) as compared to C7. G15 treatment strongly increased the mitotic activity as compared to C7 ($P<0.0001$) and C7SR ($P<0.0001$). Unlike other forms of treatment in G15 mitotic activity was constant along all parts of small intestine. Moreover, ghrelin treatment decreased BW and increased stomach weight. In conclusion, exogenous ghrelin administrated in the dose of 15 μg/kg BW for 6 days resulted in the increase of the crypt cell proliferation in the small intestine mucosa of neonatal piglets.

KEY WORDS: ghrelin, proliferation, small intestine, piglets

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INTRODUCTION

Ghrelin is 28-amino acid peptide discovered as the endogenous ligand of the growth hormone (GH) secretagogue receptor (Kojima et al., 1999). Ghrelin was shown to strongly simulate GH release *in vitro* and *in vivo* in humans and rats (Baudet et al., 2003). Although ghrelin is predominantly produced in the stomach epithelium its presence was also detected in several other tissues e.g., in the pituitary, hypothalamus, pancreas, intestine and placenta (Gualillo et al., 2001; Dembinski et al., 2003). Ghrelin exhibits broad range of biological actions such as stimulation of food intake, regulation of energy balance, control of glucose metabolism, insulin secretion (Granata et al., 2007) and modulation of cell proliferation and survival in several cell lines (Pettersson et al., 2002; Karbonits et al., 2004; Maccarinelli et al., 2005; Granata et al., 2007). In the stomach, ghrelin stimulates gastric acid secretion and gastric motility in the anaesthetized rats (Masuda et al., 2000) and exhibits gastroprotective effect (Sibilia et al., 2003). Moreover, ghrelin and its receptor expression were found in the developing gastrointestinal foetal and neonatal tissues (Rindi et al., 2002). Those data, together with substantial amounts of ghrelin present in colostrum and milk (Woliński et al., 2006) suggest the ghrelin role in postnatal development.

The aim of this study was to estimate the influence of the exogenous ghrelin on the crypt cell proliferation in the small intestine mucosa of neonatal piglets.

MATERIAL AND METHODS

The experiments and treatments were conduced in compliance with the European Union regulations concerning protection of the experimental animals. The study protocol approved by the Local Ethics Committee.

A total of 24 male neonatal piglets (Polish Landrace x Pietrain) from 12 different litters were purchased from a commercial pig farm. For the first 24 h all piglets were kept with their sows. After this period they were randomly divided into 4 groups (n=6) depending on the conditions of the further treatment. Six of them were left with their sows for the next 6 days (C7SR) and the rest were delivered to the laboratory (4 piglets for each trial) and installed in cages equipped in, so called, artificial sows - microprocessor operated system which provides equal amounts of milk replacement (Research Center Foulum-model, Pig's oline, Boss' Produkter a/s, Denmark). Milk replacer formula for piglets (protein: 19.8%; fat: 19.7%; Lakti R, Polfarma, Poland) was supplied to each piglet every 75 min (20 times per 24 h) by means of an artificial sow. Body weight was recorded every morning. The control group (C7) received milk formula and 5 ml 0.9% NaCl *via* stomach tube every 8 h. The ghrelin groups received milk formula and additionary

ghrelin (rat ghrelin, Yanaiara Institute, Japan) *via*: stomach tube in a dose of 7.5 µg/kg BW (G7.5) or 15 µg/kg BW (G15) every 8 h.
After six days of vehicle or hormone treatment the animals were sacrificed by a pentobarbiturate (Vetbutal, Biowet, Poland) overdose. GI organs (stomach, liver and pancreas) weight was recorded (Table 1) and samples of duodenum, jejunum and ileum were collected and immediately fixed in 10% neutral formalin solution.

Table 1. Body weight (kg) and organ morphometry (g/kg BW) in sow reared piglets (C7SR), fed with milk formula (C7) and supplemented with ghrelin (ZG7.5 - 7.5 µg/kg BW; ZG15 - 15 µg/kg BW)

Group	Body weight	g/kg body weight		
	kg	stomach	liver	pancreas
C7SR	2.71 ± 0.43^{a}	5.00 ± 0.54^{a}	28.02 ± 2.16^{a}	1.55 ± 0.13
C7	2.40 ± 0.46^{a}	5.97 ± 1.09	39.26 ± 6.14^{b}	1.87 ± 0.33
G 7.5	1.98 ± 0.17	$7.53 \pm 0.71^{b*}$	$28.25 \pm 3.92^{**}$	1.77 ± 0.23
G 15	$1.84 \pm 0.18^{b**}$	$6.97 \pm 0.93^{b*}$	$32.26 \pm 6.24^{**}$	1.79 ± 0.39
P	0.0009	0.0024	0.0008	0.1036

values are given as means ± SD (n=6)
^{a,b} - in the columns indicate statistical difference between groups. Kruskal-Wallis test followed by the Dunn's Multiple Comparison test. Asteriks indicate statistical difference between C7 vs G7.5; C7 vs G15 groups (Unpaired t-test or Mann-Whitney test)

To estimate the mitosis rate the slides were stained for Ki67 (Abcam GB) according to standard protocol (En Vision+ System-HRP (DAB), DakoCytomation DE). Ki67 positive cells were observed under the light microscopy (Zeiss, Germany) and 20 whole crossections per slide of (well-oriented) intestinal crypts were examined. The mitotic rate was estimated as a number of Ki67 positive cells per crypt (mean of 20 crypts)

The data are expressed as their means and standard deviation (SD). Kruskal-Wallis test followed by the Dunn's Multiple Comparison test, unpaired t-test or Mann-Whitney tests 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

Six days of ghrelin treatment resulted in a slight (G7.5) and significant (G15) reduction of body weight as compared with the control groups (C7SR, C7; Table 1). The weight of the stomach showed a significant increase in the piglets treated with both doses of ghrelin (C7SR vs C7 vs G7.5 vs G15, P=0.0024; C7 vs G7.5, P=0.03; C7 vs G15, P=0.02). Exogenous ghrelin treatments significantly decrease

the liver weights relatively to the body weight compared with C7 groups (Table 1). Was no observed ghrelin effect on the pancreas weight.

The results of mitotic index analyses are shown in Table 2. In sow-reared piglets (C7SR) mitotic index was significantly reduced in the middle and distal part of jejunum and ileum, whereas in the milk formula-fed (C7) and ghrelin-supplemented piglets (G7.5) also in the prox-jejunum. However, in the ghrelin-supplemented group (G15) mitotic index was significantly decreased in the proximal part of jejunum and very stable in all other parts of small intestine (Table 2).

Table 2. Mitotic index in duodenum, proximal, middle, distal jejunum and ileum in sow reared piglets (C7SR), fed with milk formula (C7) and supplemented with ghrelin (ZG7.5 - 7.5 μg/kg BW; ZG15 - 15 μg/kg BW)

Group	Duodenun	Jejunum			Ileum	P
		proximate	mid	distal		
C7SR	2.26 ± 1.01^{aA}	1.84 ± 0.79^{aAC}	1.78 ± 0.84^{acBC}	1.62 ± 0.86^{acBC}	1.69 ± 0.92^{acBC}	<0.0001
C7	2.32 ± 0.92^{aA}	1.26 ± 0.75^{bB}	0.99 ± 0.66^{bBC}	1.24 ± 0.76^{bB}	1.39 ± 0.95^{aBD}	<0.0001
G7.5	1.65 ± 0.73^{bcA}	1.11 ± 0.61^{bB}	1.65 ± 0.91^{acA}	0.87 ± 0.48^{bdB}	1.14 ± 0.64^{adB}	<0.0001
G15	3.32 ± 1.24^{bdA}	2.16 ± 0.92^{aB}	2.21 ± 1.00^{adB}	2.17 ± 1.04^{adB}	2.10 ± 0.87^{bB}	<0.0001
P	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	

values are given as means ± SD (n=6)

^{a,b,c,d} - in the columns indicate statistical difference between groups

^{A,B,C,D} - in the rows indicate statistical difference between the small intestine parts in each group. Kruskal-Wallis test followed by the Dunn's Multiple Comparison test

Feeding with milk formula (C7) and ghrelin supplementation in a lower dose (G7.5) reduced the mitotic index in all parts of small intestine as compared with sow reared piglets (C7SR), especially in jejunum and ileum. Exogenous ghrelin administration (G15) caused a significant increase of mitotic index (<0.0001) in all parts of small intestine as compared with other groups (C7, C7SR, G7.5).

DISCUSSION

The previous study has shown that ghrelin exhibits various actions in gastrointestinal tract of both adults and foetal. Moreover, several researches have shown that ghrelin influences cell proliferation in several cell lines. To the best of our knowledge it is the first study showing a direct stimulatory effect of exogenous ghrelin administration on the proliferation activity in the small intestine mucosa *in vivo*. This finding is crucial because in the early postnatal period crypt cell proliferation is a very dynamic process essential for changes in the crypt-villus axis structure. Moreover, we have shown that the ghrelin effect is dose dependent. The cellular and molecular action of ghrelin in the small intestine mucosa is

currently unknown. However, the researches on cell lines have shown that ghrelin up regulates cAMP which induces the cell proliferation by the activation of the Extracellular Signal Regulated Kinase 1/2 (ERK) cascade in different cell types (Baldanzi et al., 2002; Stork et al., 2002; Karbonits et al., 2004) including pancreatic β-cell lines (Granata et al., 2007).

Additionally, our present study has shown that the replacement of sow's milk (C7SR) with milk formula (C7) inhibits the mitotic activity in the small intestine mucosa. This result is consistent with our previous study (Woliński et al., 2003) on the small intestine histometry and enzyme activity and confirms that milk formula inhibits the growth of the small intestine as well as slows up the maturation of the intestinal mucosa. The significant increase in mitotic activity in G15 group as compared to C7 and C7SR suggests that ghrelin may influence maturation of the gut mucosa and requires further investigation on this subject.

Another objective of the present experiment was to determine the ghrelin effect on the body weight and organ morphometry. Although the exogenous ghrelin treatment has been shown to stimulate body weight gain in weaning pigs (Salfen et al., 2004) in our experiment we have observed an opposite effect. Those differences can be explained in several ways. Firstly, in our experiment piglets were fed *via* an "artificial sow system" providing equal amounts of milk replacement to all piglets whereas Salfen et al. (2004) experiments provided free access to water and commercial starter feed. Equal feeding procedure in our experiment was a result of a not fully developed centre of appetite and satiety in an early postnatal period. Secondly, we examined the rat origin ghrelin, which was administrated intragastric in the doses of 7.5 and 15 ug/kg BW, respectively. In Salfen et al. (2004) experiments piglets were infused *via* jugular catheter with human ghrelin in the dose of 2 ug/kg BW. Interestingly, we observed an increase in the stomach weight in both groups of ghrelin treated piglets. Stomach is a regarded as a main place of ghrelin production and ghrelin immunoreactive cells (Korbonits et al., 2004). However, in newborn humans and rodents the number of ghrelin cells in the stomach is low after birth until weaning when the ghrelin cell population is greatly expanded (Hayashida et al., 2002; Fak et al., 2007). That may explain decrease in the thickness of the stomach mucosa layer together with increase in muscularis layer observed in the ghrelin group (G15) as compared to control (C7), (Woliński et al., unpublished data). Therefore, increase in the stomach weights in the ghrelin groups (G7.5; G15) in our present experiment maybe rather a trophic effect of peripheral action of the ghrelin administrated to the stomach in this stage of piglet's development.

CONCLUSIONS

In conclusion, our results show that ghrelin stimulates crypt cell proliferation in the small intestine mucosa. Moreover ghrelin treatment resulted in a slight

(G7.5) and significant (G15) reduction of body weight as compared with the control groups (C7SR, C7). However, we observed a significant increase in the stomach weight in the piglets treated with both doses of ghrelin. All together suggest ghrelin multiple action in gastrointestinal track of neonatal piglets.

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