THE EFFECT OF SUPPLEMENTING SOW WITH BIOACTIVE SUBSTANCES ON NEONATAL SMALL INTESTINAL EPITHELIUM

Department of Physiological Sciences and Department of Clinical Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, Warsaw, Poland

Development of the small intestinal epithelium in early postnatal period has a significant influence on pig's survival rate and further productivity. The aim of this research was to verify whether the diet supplementation of pregnant and lactating sow with a blend of bioactive substances (flax seed, rapeseed, linden inflorescence, taurine, L-carnitine and tocopherol acetate) had an effect on the development of intestinal epithelium in their offspring. The doses of bioactive substances were calculated to meet the demands for optimal supply of the pig fetuses and newborns. Pig neonates from two groups of sows, control and supplemented, were sacrificed at the day 1, 2, 4, 7 and 14 of life. The samples taken from mid-jejunum were evaluated for mitosis (Ki67), apoptosis (active caspase 3), autophagy (MAP1 LC3), and DNA damage (p53). Increase of mitotic index was noticed at day 1, 4 and 7 for supplemented group when compared to the control. Reduction of apoptotic index was observed at day 2 as compared to control. A tendency toward elevated autophagy was observed during the first 2-4 postnatal days in both groups. p53 expression was significantly lower in supplemented group as compared to control. Overall, the mitosis to programmed cell death ratio was increased and the maturation of epithelial cells quickened. We suppose that the supplementation of pregnant and lactating sow diet with bioactive substances enhanced maturation of the small intestinal epithelium in their offspring during the early postnatal period.

Key words: mitosis, apoptosis, autophagy, mucosa remodeling
INTRODUCTION

The development of the small intestinal mucosa in the fetal and early postnatal period is very intensive and requires, besides structural compounds and energy, a great number of substances with minor nutritive value but of relevant biological activity. These bioactive substances are supplied by the mother to the fetuses by placental circulation, and to the newborns with colostrum and milk. Contemporary feeding strategies reduced or totally eliminated a great number of bioactive substances from diets such as polyunsaturated fatty acids (PUFA) from n-3 family, plant polyphenols and other antioxidants, vitamin-like compounds: taurine and carnithine in farm animals as well as in humans (1-3). On the other hand, intensified development of fetuses and newborns substantially increased the demands for bioactive substances. Among mammalian species, the fast growing sow fetuses and newborn pigs is regarded a good experimental model. The knowledge of the importance of single biologically active substances and their groups in the perinatal development is rapidly increasing (2, 4-6). Based on literature survey, we composed a blend of bioactive substances and plants to supplement the standard sow diet with selected bioactive substances which seem to be particularly deficient, and on the other hand seem to be important in controlling the perinatal development in pigs. The idea was to modify their concentrations in the sow plasma and milk in order to meet the demands of fast-growing pig fetuses and neonates. The most important changes in gastrointestinal tract, induced by the change to the intestinal nutrition after birth, take place in small intestinal epithelium. They are the growth of mucosa layer and enterocyte maturation, associated with the decline of apical canalicular system (ACS) in the enterocytes. Dramatic growth of the mucosa in early life is a result of collostral molecule incrustation to the enterocytes (via ACS), as well as an increase in cell number (7-9). The increase in cell number is controlled by dynamically changing equilibrium between proliferation (mitosis) and programmed cell death (PCD) - apoptosis and autophagy, especially in the small intestine, where processes of cell elimination and replacement are quick and intensive.

The aim of the present study was to investigate the effect of supplementation a blend of bioactive substances fed to sows on the development of small intestinal epithelium in their offspring. In preliminary studies we quantitatively evaluated the mitotic, programmed cell death (PCD) apoptotic and autophagy indexes, and the extent of DNA alterations in healthy small intestine mucosa of control rats. According to our findings and previous reports on piglets and rats (8, 10), we designated the middle part of the jejunum the best for the analysis. In that part of gastrointestinal tract a relatively small variability in the gut lumen conditions associated with temporary fluctuations in digestive secretions, pH, water-
electrolyte balance and nutrients flow reflected on the smallest differences between studied animals within experimental group.

MATERIAL AND METHODS

Animals and study protocol

Animal studies were approved of by the Local Ethical Committee. A total of 12 pregnant sows (Polish landrace x Pietrain) were used in the study. At day 80 of the pregnancy the sows were randomly divided into 2 groups, control (n=6) and supplemented (n=6). The sows from the control group were fed with the standard diet for pregnant (DM 87.6%, ME 11.35 MJ/kg, CP 13.1%) and lactating (DM 87.3%, ME 12.93 MJ/kg, CP 15.4%) sows. The sows from the supplemented group received the standard diet for pregnant and lactating sows supplemented with a blend (Table 1) of substances - taurine (Otis, Poland), L-carnitine (Lonza, Poland), tocopherol acetate (Sigma-Aldrich, Poland), and plants - flaxseed and rapeseed providing with α-linolenic (C18:3n-3) and linoleic (C18:2n-6) fatty acids, and linden inflorescence (Kawon, Poland) as a source of flavonoids and other antioxidants, e.g. phenolic acids. For the supplemented sows the original composition of the control diet was modified to obtain an energy and protein content in the supplemented diet similar to that in the control diet. Blood plasma, colostrum and milk analysis demonstrated substantial transfer of n-3 fatty acids, plant polyphenols and other antioxidants from the supplemented diet into sow blood and milk, only plasma and milk concentrations of carnitine was not influenced (11, 12). One piglet from each litter was sacrificed for tissue sampling on postnatal days 1 (unsuckling newborns), 2 (24 h after birth), 4, 7, and 14. The whole tissue sections of the mid-jejunum were embedded in a freezing medium, then frozen in the liquid nitrogen and stored at -80°C.

Staining and analysis

Slices of intestine samples (15 µm) were rinsed with PBS and labeled with the specific sets of antibodies. For mitosis analysis, an anti-Ki-67-FITC-conjugated antibodies (BD Pharmingen, San Diego, CA, USA) was used. The Ki-67 protein is an absolute requirement for cell progression through the division cycle as it organizes chromatin structure. It is expressed in every phase of cell-division cycle except G0 phase thus allowing quantification of whole population of dividing cells. To measure apoptosis, an anti-Cpp32 fragment of active caspase 3 (DAKO, Glostrup, Denmark),

Table 1. The composition of bioactive substances per 1 kg of feed used to supplement the standard diet for sows from the supplemented group.

<table>
<thead>
<tr>
<th>Component</th>
<th>Pregnant</th>
<th>Lactating</th>
</tr>
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<tbody>
<tr>
<td>Flax (Linum usitatissimum L.) seed, g</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Rapeseed, g</td>
<td>40</td>
<td>40</td>
</tr>
<tr>
<td>Linden (Tilia cordata) inflorescence, g</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Taurine, mg</td>
<td>500</td>
<td>1000</td>
</tr>
<tr>
<td>L-carnitine, mg</td>
<td>50</td>
<td>120</td>
</tr>
<tr>
<td>Tocopherol acetate, mg</td>
<td>150</td>
<td>150</td>
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</table>
secondary swine anti-rabbit FITC-conjugated antibodies (DAKO, Glostrup, Denmark) were employed. Active caspase 3 is a major effector caspase involved in execution of the irreversible phase of apoptosis. For autophagy analysis, an anti-MAP I LC3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used along with secondary antibodies - Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA). MAP I LC3, the only reliable marker of PCD II, occurs on the circumference of nucleus during amino-acids starvation and is involved in formation of the autophagosome membranes. DNA damage was evaluated using anti-p53-FITC-conjugated antibodies (DAKO, Glostrup, Denmark). The p53 protein is expressed in the case of DNA damage and facilitates "cell cycle arrest" in a G1 phase allowing either the repair of DNA or elimination of the damaged cell. Afterwards cell nuclei were counterstained with 7 aminoactinomycin D (7AAD) (Sigma-Aldrich Corporation, St. Louis, MR, USA).

Images were acquired with the use of FV-500 laser scanning confocal microscope (Olympus Polska sp. z o.o., Warsaw, Poland), and quantitatively analyzed using the Microimage - image analysis software (Olympus Polska sp. z o.o., Warsaw, Poland). At least 15 images were analyzed for each data point. The values are given as geomeans and standard error of mean (SEM). The statistical analysis was made using unpaired Student t-test and one-way analysis of variance (ANOVA) followed by Tukey test and test for linear trend as appropriate (GraphPad PRISM, USA).

RESULTS

The average number of delivered piglets in the control and supplemented group was not different. At birth the piglets from control group showed a tendency toward larger body weight as compared to supplemented group, however, the differences were reversed on the 4th postnatal day (11).

The mitotic index in control piglets showed no statistically significant variations during the study period (Fig. 1). However, in the supplemented group the differences were significant, and the highest mitotic index was observed in days 1, 4 and 7. What is more, between days 2, 4 and 7 the linear trend of increase was found (p=0.018). On day 14, the mitotic index in supplemented group was significantly reduced as compared to values of earlier samplings (Fig. 1A). The apoptotic index did not show significant variations in the both groups, besides a difference (p<0.05) at day 2 of life (Fig. 2B). Mitosis to apoptosis ratio in the control group was 0.69, 0.34, 0.29, 0.36, 0.52 in the day 1, 2, 4, 7 and 14 of life, respectively. During the first postnatal week the mitosis to apoptosis ratio in the supplemented group was higher (respectively, 0.87, 0.47, 0.73, 0.83) as compared to control. Interestingly, the largest difference (2.3 - 2.5 fold) was observed in days 4 and 7. In the 14 day old piglets from supplemented group the ratio was 0.35, thus lower than in the control.

The expression of MAP I LC3 in control group did not differ during the entire study period (Fig. 1C). However, in the supplemented group the expression in day 2 was significantly larger than that in day 4. Figure 2 shows cells co-expressing MAP I LC3 and active caspase-3 as well as cells expressing only MAP I LC3 located in the apical part of the cell body. This pattern was not
observed in older piglets, i.e. 7 to 14 days old controls and 4 to 14 days old supplemented piglets (Fig. 1C).

The expression of p53 in the intestinal epithelium of supplemented piglets was significantly lower in day 1, 2 and 7 of life as compared to the control (Fig. 1D).
Furthermore, there were statistical differences in the control piglets during the study period, but not in the supplemented.

**DISCUSSION**

The most noticeable differences between the control and supplemented group were observed in the mitotic index, mitosis to apoptosis ratio and p53 expression. In accordance to the increase in crypt cell number an increase in daily growth rate of supplemented piglets was observed. The piglets from supplemented group tended to have a little less weight at birth than the controls, what presumably can be related to the differences in placental supply with bioactive substances. However, along with the start of suckling these piglets grew faster than the controls, thus the supplementation of sow diet as well as the changes found in the gut epithelium were considered beneficial for the offspring development.

The control values of mitotic index corroborate with the values observed in neonatal gut mucosa elsewhere (8). But the supplementation of sow diet used in this study significantly increased them. Tissue homeostasis depends on the balance between mitosis and programmed cell death (mostly apoptosis). Change towards proliferation entails increase of the weight of intestine, observed particularly during the first few days of life (8). Since the apoptotic index was not different between control and supplemented piglets (besides day 2 of life) the blend of bioactive substances used in this study seemed to have the pro-mitotic effect rather than the anti-apoptotic. Concerning the apoptosis, the expression of active caspase-3 was observed all along the villi as well as in the crypts (*Fig. 2B*), what is characteristic for newborns (8, 13). The apoptotic cells often died in
groups (packets). Contrastingly, in mature animals and humans the single cells dying of apoptosis are present on the upper one-third of the villi (14, 15).

Autophagy seemed to be a stable process in small intestinal epithelium (8, 10). Surprisingly, it was not the case in our newborns in which the fetal-type enterocytes expressed MAP I LC3 not only in the phagosomes but also in the top of the cell. The cellular microstructures expressing MAP I LC3 resemble microvacuoles belonging to the apical canalicul system, ACS (7). Although MAP I LC3 is regarded to be a good marker of autophagy (16), it needs to be used with caution in gut epithelium of neonates. Nevertheless, the ACS stained with MAP I LC3 helps to differentiate between fetal- and adult-type enterocytes. In our study, the decrease in MAP I LC3 expression was found in supplemented group earlier than in the control suggesting faster maturation. Figure 1C presents microphotographs of fetal-type enterocytes with ACS stained with MAP I LC3 (left) and mature enterocytes lacking ACS with autophagosomes stained (right).

The expression of p53 in the gut mucosa is very low, besides the area of intensive cells divisions (crypts), and cells with damaged DNA (8, 17). Interestingly, in our study p53 expression was extremely low in the supplemented group, possibly due to the abundance of antioxidants, omega-3 acids and taurine supplemented with mother's milk that might reduce oxygen free radicals (11, 12, 18). Such low p53 expression indicates low number of defectively divided cells that have to be eliminated and in consequence increased pool of fully functional young epithelial cells.

In conclusion, the present study suggests that the introduction of a blend of biologically active substances into the sow fed stuff results in sped up remodeling of the gut epithelium presumably due to modification of fetal and neonatal supply with bioactive substances. The remodeling was accompanied with less DNA damages.

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Author’s address: Michał M. Godlewski, Ph.D., DVM, Department of Physiological Sciences, Faculty of Veterinary Medicine, Warsaw Agricultural University, Nowoursynowska 159, 02-776 Warsaw, Poland; Phone/Fax: +48 22 8452472; e-mail: mickgodl@hotmail.com