Effects of Single or Combined Supplementation of Probiotics and Zinc on Histological Features of Ileum, Glucagon Like Peptide-1 and Ghrelin Levels in Malnourished Rats

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Abstract

Introduction: Malnutrition causes small intestinal atrophy leading to impaired nutrient absorption, zinc deficiency, and intestinal microbiota imbalance affecting appetite. Zinc-only supplementation programs have been shown to be ineffective in reducing the national prevalence of malnutrition.

Aim: The aim of this study was to assess the effects of probiotics and zinc in single or combined supplementation on the histological features of ileum and appetite regulating hormone in malnourished rats.

Materials and methods: This study included 25 rats aged 8 weeks (weight 150-200 g) divided into 5 groups. Group A served as normal receiving standard diet, group B served as malnourished receiving low calorie diet. Groups C, D, E were pretreated with calorie restriction for 14 days to induce malnutrition. The treatment was given for 14 days. Group C was treated with probiotics and zinc combination, group D was treated with probiotics, group E - with zinc. All treatment groups received standard diet at the same time. Ileum sample was taken and subjected to histological preparations using hematoxylin-eosin staining to evaluate villi height and mucosal thickness, blood sample was taken for GLP-1 and ghrelin levels evaluation using ELISA methods.

Results: Probiotics and zinc co-supplementation significantly increased villi height and zinc supplementation significantly increased mucosal thickness compared to malnourished rats. GLP-1 levels were significantly increased and ghrelin level was reduced with single or combined supplementation of probiotics and zinc.

Conclusions: Low-calorie feed administered within 14 days successfully changed the profiles of small intestinal histology in rats. Either single or combined administration of probiotics and zinc develop the histological features of ileum and appetite in the malnourished rats.

Keywords

appetite, malnutrition, nutrient absorption, probiotics, zinc, rats
INTRODUCTION

Malnutrition causes small intestines atrophy inhibiting the function of nutrients absorption. This atrophy results in zinc deficiency and intestinal microbiota imbalance affecting appetite. Zinc supplementation programs have been shown to be ineffective in reducing the national prevalence of malnutrition. Data in Indonesia showed that the number of children under five years of age with moderate and severe undernutrition reached 17.7% which remain below the target of 17% and children under two years old with stunting and severe stunting reached 29.9% while the target was 28%. Although malnutrition is not an infectious disease, it caused lethal outcome in children in more than 50%. Chronic malnutrition can lead to stunting and affects cognitive achievements and national problems in the future due to low economic productivity. Human studies are obviously limited in terms of investigating gut function in malnourished children, in particular because of the lack of precise non-invasive methods. Animal models, therefore, offer an affordable tool to better investigate the mechanisms associated with malnutrition and highlight potential biomarkers which can be translated back to the clinical condition.

The small intestinal damage frequently results in a gastrointestinal tract infection. Besides, the infection also results in decreasing the appetite which affects nutritional metabolism and utilization. The nutritional status of children with malnutrition in a balance condition of negative energy (catabolism) affects the production of hormones in the digestive tract, such as GLP-1 and ghrelin. The small intestinal structure repair is very much needed to improve the absorption of nutrients. Probiotics and zinc are recognized as having the ability to reduce the damage severity of microvilli by maintaining the integrity of intestinal mucosa, improving immunity, and as immunomodulation. The other essential role of probiotics is to improve people’s appetite by improving the production of short chain fatty acids (SCFAs) derived from bacteria fermentation. Zinc serum level is found low in children with malnourished protein energy. The zinc supplementation during the rehabilitation phase is closely related to the physical weight gain, might significantly improve growth as well as decrease stunting prevalence, less nutrition, anemia in children, and might also improve appetite. Previous research concluded that probiotics were greatly beneficial to repairing the intestinal microflora balance and improving the villi height in ileum of rats experiencing diarrhea after the infection of Escherichia coli.

AIM

This study was designed to assess the effects of probiotics and zinc supplementation on the histological changes of ileum and appetite regulating hormone by looking for GLP-1 and ghrelin levels in malnourished rats.

MATERIALS AND METHODS

The study was conducted from October to November 2019 in the experimental laboratory Center Food and Nutrient, Universitas Gadjah Mada Yogyakarta for the intervention and blood analysis. Paraﬃn blocks and hematoxylin-eosin staining were done in the Pathological Anatomy Laboratory, Sultan Agung Islamic Hospital of Semarang. The protocol was approved by the Medical Ethics Committee, Faculty of Medicine, Universitas Islam Sultan Agung with number 603/VIII/2019/Komisi Bioetik.

Experimental animals

Twenty-five 8-week-old Wistar male rats, weighing 150-200 grams, were randomly divided into ﬁve groups, 5 animals in each (according to World Health Organization guidelines). Group A served as control receiving standard diet, group B served as malnourished group receiving low calorie diet. Groups C, D, E were pretreated with calorie restriction for 14 days to induce malnutrition.

The animals for all groups had free access to water. All animals were kept under standard individual housing conditions: living space 750 cm², temperature 22±2º, and 12/12 h light/dark photoperiod cycles.

Precondition treatment

In this study we used the malnourished rats by be feeding them with low-calorie food in the dosage of 4 g/100 grams of body weight/day for 14 days before the research started.

Intervention

The treatment was given for 14 days. Group C was treated with probiotics and zinc combination, group D was treated with probiotics only, group E was treated with zinc only. All treatment groups received standard diet at the same time. At the end of the study, all rats were anesthetized with an intramuscular injection of 50 mg/kg ketamine hydrochloride (Ketalar).

Probiotics Dosage

The probiotics used were composed of tyndallized lyophilisate Lactobacillus acidophilus and Bifidobacterium longum. The dosage of probiotics was due to the conversion of adult’s body weight (70 kilograms) to the rat’s body weight (200 grams) by 6.12 milligrams/day administered with the frequency of once per day for 14 days with oral gavage.

Zinc Dosage

Zinc tablets are dissolved in water. The dosage of zinc was due to the conversion of adult’s body weight (70 kg) to the rat’s body weight (200 grams) by 0.36 milligrams/day administered with the frequency of once per day for 14 days with oral gavage.
Histological features of ileum

Ileum sample was taken and subjected to histological preparations using hematoxylin and eosin staining to evaluate the villi height and mucosal thickness within µm unit seen through observations utilizing microscopes with the magnification of ×400 and calibrated using the OptiLab 2.1 Image Raster software.

Hormone levels

Blood samples were collected by puncture of sinus orbitalis and taken for GLP-1 and ghrelin levels evaluation using ELISA methods within pg/mL. The ELISA test was performed according to manufacturer recommendations (Rat Ghrelin ELISA Kit, ABclonal, Rat GLP-1 ELISA Kit, FineTest®).

Statistical Analysis

Data were expressed as means ± standard deviation. Data obtained from the research result were then analysed using the normality test of Shapiro-Wilk test and homogeneity of variance test with Levene's statistics and were found to be normal and homogenous (p>0.05). ANOVA test was used to analyze the differences found in the treatment groups and to figure out the differences between groups with LSD test. The data analysis process was conducted using SPSS 22.0 for Windows. The decision to accept or refuse the hypothesis was based on the value of α - 5% (p<0.05).

RESULTS

After 14 days’ treatment of combined probiotics and zinc supplementation, the result of villi height, mucosal thickness, GLP-1 and ghrelin levels assessment can be seen in Fig. 1, and Table 1.

Effects of probiotics and zinc on:

Villi height

The highest villi were in group A (normal group) (302±39 µm), followed by group C (probiotics + Zn) (256±35 µm), group E (zinc) (246±42 µm), group D (probiotics) (210±44 µm),
μm), and group B (malnourishment) (200±28 μm). ANOVA statistical analysis showed that there was significant difference between groups (p=0.003). Moreover, the post hoc test indicated that villi height in normal and combined groups was significantly higher compared to malnourished group (p<0.05). Whereas the villi height in probiotics and zinc groups was not significantly different compared to malnourished group (p>0.05) (Fig. 1).

**Mucosal thickness**

The highest mucosal thickness was found in group A (273±64 μm), followed by group E (239±47 μm), group D (169±30 μm), group B (155±16 μm), and group C (152±32 μm). One-way ANOVA statistical analysis demonstrated that there was significant difference between groups (p<0.001). Moreover, the post hoc test indicated that mucosal thickness in normal and zinc groups was significantly higher compared to malnourished group (p<0.05). Whereas the mucosal thickness in combined and probiotics groups was not significantly different compared to malnourished group (p>0.05) (Fig. 1).

**GLP-1 levels**

The highest level of GLP-1 was in group A (19.8±1.2 pg/ml), followed by group D (15.9±0.8 pg/ml), group E (15.4±0.9 pg/ml) and group C (11.5±0.5 pg/ml), and the lowest was in group B (5.3±0.6 pg/ml). The result of one-way ANOVA statistical analysis indicated that there was significant difference amongst groups (p<0.001). The post hoc test showed that GLP-1 levels in all groups were significantly higher compared to malnourished group (p<0.05) (Fig. 1).

**Ghrelin levels**

The lowest level of ghrelin was found in group A (59.9±1.9 pg/ml), followed by group C (72.9±3.2 pg/ml), group D (80.3±3.5 pg/ml) and group E (87.2±1.3 pg/ml), and the highest was in group B (164.5±8.0 pg/ml). One-way ANOVA statistical analysis indicated that there was significant difference amongst groups (p<0.001). The post hoc test indicated that ghrelin levels in all groups were significantly lower compared to those in the malnourished group (p<0.05) (Fig. 1).

### DISCUSSION

The result of the conducted research showed that there were villi damage and thinning small intestinal mucosa in the malnourished rats group (Fig. 2). It also showed that the low-calorie food administered for 14 days successfully changed the profiles of small intestinal histology. The result of this research was in accordance with the previous one showing that malnourished condition in the experimented rats might worsen the small intestinal morphology. Some research explained that the small intestinal damage in malnourished condition results in the disturbance of digestive enzyme function, microvilli composer's expressions, and protein signalling pathways. The malnourished condition results in the intestinal mucosa atrophy as the body's response to the nutrient deficits. The intestinal mucosa cells reduced their sizes to maximize their functions and survival purposes while the villi height in small intestine also decreased. The small intestinal villi atrophy was caused by the shortening crypt due to the decreasing mitosis activity caused by stem cells located on the crypt base replacing the dead cells. Besides, low protein and DNA level might cause crypt experience hypoplasia.

The treatments administered for 14 days to all groups showed the developing villi height. The previous study explained that the developing villi height in malnourished condition showed that the muscle tissues and blood vessels forming the intestinal wall were greatly affected by the number of consumed nutrients. More nutrients are consumed make more active the intestine perform the digestive activities that the growth of digestive organs will be also well stimulated. In contrast, in the malnourished group, the less the number of nutrients is consumed, the movement of intestine will also decrease (hypo motility) and cause villi atrophy. The developing villi height in the treatment groups improve the nutrition status since the nutrients absorption process in the ileum villi increased. Better nutrients absorption could also make the number of granulated convoluted tubules (GCT) cells found in the rats' glandula submandibula increased. These cells contained active peptides, such as EGF and NGF which had the ability to improve the epithelium cells' proliferation activity and mitosis.

### Table 1. Mean villi height, mucosal thickness, GLP-1 and ghrelin levels

<table>
<thead>
<tr>
<th>Variables</th>
<th>Groups</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A (n=5)</td>
<td>B (n=5)</td>
</tr>
<tr>
<td>Villi height</td>
<td>302.2±39.0</td>
<td>199.6±28.3</td>
</tr>
<tr>
<td>Mucosal thickness</td>
<td>273.2±64.3</td>
<td>155.2±15.5</td>
</tr>
<tr>
<td>GLP-1 levels</td>
<td>19.8±1.2</td>
<td>5.3±0.6</td>
</tr>
<tr>
<td>Ghrelin levels</td>
<td>59.9±1.9</td>
<td>164.5±8.0</td>
</tr>
</tbody>
</table>

*ANOVA test
The proliferation process of the small intestinal villi was related to the benefit of probiotics and zinc found in the previous research. Probiotics decrease the villi damage and increase the small intestinal mucosal thickness after the infection of Enteropathogenic \textit{E. coli} (EPEC).\textsuperscript{15} Probiotics and zinc maintain the integrity of the intestinal mucosa by improving the number of \textit{tight junction} protein that the cells’ permeability decreased and the cells became more selective in receiving substances or unrecognized objects. The increasing number of tight junction was related to the increasing expressions of ZO-1, ZO-2, and claudin.\textsuperscript{16} The growth of intestinal epithelium cells stimulation was affected by the fermentation process produced by the SCFAs. These SCFAs were produced by \textit{Bifidobacteria} and \textit{Lactobacilli} that stimulate the growth of intestinal epithelium cells and increase the intestinal villi height.\textsuperscript{15}

\textbf{Figure 2.} The staining results of hematoxylin-eosin (magnification $\times400$) to see the ileum villi height, group A, B, C, D, and E. The ileum mucosal histological profile of malnourished group (B) showing the unequally distributed villi height and the decreasing mucosa thickness. This shows a severe erosion process. Description: the red arrow shows villi height, the black arrow shows mucosal thickness.
This research showed that administration of zinc was better in improving the small intestinal mucosal thickness when compared to the other treatments. Malnourished condition result in zinc deficiency as well as not maximally expressed ZO-1 and occludin protein that the administration of only probiotics not maximize the ileum epithelial proliferation when compared to zinc group. Besides, the characteristics of probiotics which were uneasy to evaluate had come into the digestive tract. It was difficult to make sure where prebiotics will colonize and how was the stabilization of colonies formed. The colonies were mostly found in the large intestine than in the small one. The previous research result showed that the administration of zinc supplementation improves the small intestinal epithelium by increasing the proliferation of cells. Zinc was a transporter in the cellular level metabolism and had a receptor in each mucosa cell that the expressions of occludin and ZO-1 protein in the group administered either with only probiotics or zinc affect the function of TJ in both groups. The expressions from the zinc transporter of ZnT1 were affected by the zinc supplementation help the mediation process of zinc transportation from the intestinal epithelium to extracellular.

It has been previously explained, the integrity of small intestinal mucosa is greatly related to the hormone secretion which affect appetite. This research showed that the administration of probiotics and zinc in both forms of combination or single increase the GLP-1 level when compared to the malnourished group. This was in accordance with some research explaining the metabolism changes of amino acids and fatty acids in malnourished condition might change the hormone level and growth factors, such as ghrelin, GH, insulin, cortisol, and IGF-1, while for the mechanism of GLP-1 and PYY in the case of malnutrition had not been completely understood. The major stimulus of GLP-1 hormone was derived from the nutrients intake and would be released after the nutrients were digested, This GLP-1 would be rapidly secreted from the L-enteroendocrine cells. Probiotics produce SCFAs which was able to stimulate the small intestinal enteroendocrine cells to release the GLP-1 hormone. SCFAs stimulate the GPR43 in the intestinal mucosa to induce the secretion of GLP-1. Besides, probiotics produce metabolites, such as indole and it derivatives, as well as tryptamine. This indole could maintain the tight junction in the small intestinal mucosa and stimulate the L enteroendocrine cells to release GLP-1.

Zinc is known to have different mechanism when compared to probiotics to activate GLP-1. The previous research proved that GLP-1R had been closely related to ZIMIR-Ex4, required Zn\textsuperscript{2+}, and eventually released GLP-1. The other mechanism from zinc is through activity induction of GPR39 by Zn\textsuperscript{2+} located in GLP-1. This research proved that the secretion of high GLP-1 found in the treatment group was due to the more absorbed nutrients and reciprocal relationship in the proliferation of small intestinal villi.

The other affected hormone resulted from the developed small intestinal villi function was ghrelin. The research showed that the highest ghrelin level was in malnourished group since the nutrients absorption ability was not as good as that in the other groups. The highest ghrelin level was also found in patients with malnourished condition and not given any nutritional therapy when compared to those administered with the therapy. The research on ghrelin in obesity condition showed the contradiction, in which the ghrelin synthesis and secretion in rats decreased. Ghrelin was produced by the stomach and affected by the absorbed nutrients and hormonal factors. Ghrelin level rapidly decrease after the body consumes food. The administration of probiotics and zinc in each group affected the small intestinal villi to develop that the nutrients were more maximally absorbed and gave different hormonal responses. The result of ghrelin level was in accordance with that of histological profile changes of each group. This research proved that the better the ileum villi were developed, the better the nutrients were absorbed that the ghrelin hormone released to the blood circulation.

**CONCLUSIONS**

Low-calorie food administered within 14 days successfully changed the profiles of small intestinal histology in rats. This research showed that either single or combined administration of probiotics and zinc developed the histological features of ileum and appetite in the malnourished rats. The effectiveness difference between treatment groups might be figured out by looking at the expressions from ZO-1, ZO-2, and claudins which had the role to maintain the tight junction, while the number of GLP-1 receptors and ghrelin producing cells had been administered with probiotics and zinc. More work needs to be done to understand this.

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**Conflict of Interest**

The authors report no conflicts of interest.

**Author disclosure**

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Влияние однократного или комбинированного приёма пробиотиков и цинка на гистологические характеристики подвздошной кишки, уровни глюкагоноподобного пептида-1 и грелина у истощённых крыс

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Резюме

Введение: Недоедание вызывает атрофию тонкого кишечника, что приводит к нарушению всасывания питательных веществ, дефициту цинка и дисбалансу кишечной микробиоты, что влияет на аппетит. Программы с добавками только цинка оказались неэффективными в снижении распространенности недоедания в нашей стране.

Цель: Целью этого исследования было оценить влияние пробиотиков и цинка в качестве одной или комбинации добавок на гистологические характеристики подвздошной кишки и гормона, регулирующего аппетит, у истощённых крыс.

Материалы и методы: В исследование были включены 25 крыс в возрасте 8 недель (вес 150-200 г.), разделяённых на пять групп. Группа А включала крыс на стандартной диете с нормальным потреблением, группа Б – истощённых крыс на низкокалорийной диете. Группы В, Г и Д не получали предварительно ограниченную диету в течение 14 дней, чтобы вызвать недоедание. Курс лечения длился 14 дней. Группа В получала комбинацию пробиотиков и цинка, группа Г – только пробиотики, а группа Д – только цинк. Все исследуемые группы в то время находились на стандартной диете. Боль на образцы подвздошной кишки и обработаны гистологическими образцами путём окрашивания гематоксилин-эозином для оценки высоты ворсинок и толщины слизистой оболочки, а также взят образец крови для оценки уровней GLP-1 и грелина с помощью ELISA.

Результаты: Совместное введение пробиотиков и добавок цинка значительно увеличивало высоту ворсинок, добавление цинка значительно увеличивало толщину слизистой оболочки по сравнению с таковыми у истощённых крыс. Уровни GLP-1 были значительно повышены, а уровни грелина были снижены при приёме только одной или комбинации пробиотических добавок и добавок цинка.

Заключение: Снижение потребления калорий в течение 14 дней успешно изменяло гистологические профили тонкой кишки у крыс. При однократном или комбинированном введении пробиотиков и цинка у истощённых крыс развивались гистологические характеристики подвздошной кишки и аппетита.

Ключевые слова
аппетит, недоедание, всасывание питательных веществ, пробиотики, цинк, крысы