



The Effect of Glutamine Supplementation on Ileum Histopathology of Rats with Acute and Chronic Enteropathogenic *Escherichia coli*-induced Diarrhea

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Abstract

BACKGROUND: Glutamine is a non-essential amino acid as the main fuel in the gastrointestinal mucosa. By its various gastrointestinal functions, glutamine is thought to increase the protection of the intestinal mucosa against local or systemic injury from diarrhea.

AIM: This study aims to determine the relationship between glutamine supplementation and ileum histopathology in rats with acute and chronic diarrhea induced by enteropathogenic *Escherichia coli* (EPEC).

MATERIALS AND METHODS: The research was conducted in an experimental laboratory with a randomized posttest-only control group design. A total of 30 *Rattus norvegicus* strain Wistar were divided into five groups. The treatment group was induced to have diarrhea using EPEC at a dose of 108 CFU/ml, after each group diagnosed by acute and chronic diarrhea followed by glutamine supplementation at a dose of 810 mg/200 g for 14 days. The intestinal histopathology of each group was assessed and their levels of inflammation classified based on the Barthel-Manja inflammatory score.

RESULTS: The result showed that significant differences in inflammation levels between the negative control group, acute diarrhea group, chronic diarrhea group, acute diarrhea group with glutamine, and chronic diarrhea group with glutamine were found ($p < 0.05$). The highest level of inflammation was found in the acute diarrhea group.

CONCLUSION: This study concluded that glutamine supplementation has an effect on the ileum histopathology of rats with acute and chronic diarrhea.

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Introduction

Diarrhea is a condition in which stools are passed with a watery consistency that usually occurs with a frequency of more than 3 times/day [1]. Diarrhea is a major cause of morbidity and mortality in children worldwide, where 98% of deaths occur in developing countries, including Indonesia [2], [3]. Results of the 2013 Indonesian Basic Health Research stated in Riau Province, diarrhea was the second most common cause of toddlers' mortality, accounting for 17.2% of all toddler deaths [4]. The choice of area of Riau was because researchers worked in the largest public hospital in Riau Province.

A strain of *Escherichia coli* that can infect and cause diarrhea, especially in children in developing countries, is enteropathogenic *E. coli* (EPEC) [5], [6]. The mechanism by which EPEC causes diarrhea starts with the process of attaching and effacing. This

pathogen can attach to intestinal epithelial cells and destroy (efface) the microvilli, producing attaching and effacing lesions (A/E lesion). EPEC, which causes cytoskeletal changes, can damage the cytoskeleton border under the microvilli membrane and cause the proliferation of actin filaments. In severe infections, it can totally destroy the absorption membrane of the intestinal surface, villous atrophy, and thinning of mucous membranes. As a result of changes in shape to be like bound piles, EPEC can form a microcolony which produces a localized adherence pattern. This damaged epithelial structure results in the decreased absorptive capacity of the intestinal mucosa and loss of tight junction integrity that leads to reduced epithelial resistance, increased membrane permeability, disruption of Na + K + pumps, and inhibition of water and electrolyte absorption which causes diarrhea [7].

This mechanism of inflammation caused by EPEC renders damage to the small intestine's histopathology [7]. In previous studies, histological

changes in the small intestine were found to occur in diarrhea, both in acute diarrhea and chronic cases. Changes that occur in the intestinal tissue are used as a step in the diagnosis and evaluation of therapy of diarrhea by examining the degree of inflammation and damage to the mucosa [8], [9].

Glutamine is a non-essential amino acid with an abundant amount in the human body. In the gastrointestinal tract, glutamine is known as the main fuel of the intestinal mucosa that triggers enterocyte growth, increases intestinal barrier function, increases blood circulation to the intestine, plays an important role in the synthesis of nucleic and amino acids in the intestinal barrier, maintains integrity of the tight junction, and acts as an immunomodulator. Glutamine is produced by the body in sufficient quantities, but its use tends to increase during illness including diarrhea, which causes glutamine depletion. This condition will interfere with the functions of glutamine and aggravate an illness. Diarrhea will be followed by damage to the intestine and glutamine deficiency will inhibit its repair. Therefore, the supplementation of exogenous glutamine is needed to help the regeneration of damaged intestinal mucosa [10].

The benefits of glutamine as an adjuvant in managing diarrhea have been obtained based on several studies. A study by Rao Radha, Maria Vicario, and Carneiro-Filho showed that glutamine has protective properties for mucosal barriers due to its effects on maintaining the integrity of intestinal epithelial tight junction cells [11], [12], [13]. In addition, glutamine could also facilitate enteral absorption of nutrients and electrolytes in diarrhea and reduce the severity of diarrhea by increasing water and salt uptake [14].

Based on a previous study, glutamine can improve intestinal growth, promote enterocyte proliferation and survival, and regulate the intestinal barrier function during injury, infection, stress, and catabolic conditions. This effect occurs because glutamine can maintain intracellular redox status and regulate gene expression associated with various signaling pathways [15]. Research from Roedi Irawan also supported that glutamine could repair ileal microvilli in malnourished rats. In addition, an increase in intestinal enzyme activity, namely, sucrase, maltase, lactase, spectrin, as well as clathrin, was found. This growing evidence supports the idea that glutamine is beneficial for intestinal repair [16]. Furthermore, a study by Huang showed the beneficial of glutamine on improvement of *Rattus norvegicus* strain Wistar's intestinal that is experiencing chronic diarrhea [17]. However, different results are shown from the study by Kamucakhi about the benefits of glutamine supplementation in children who experience chronic diarrhea, in which glutamine supplementation showed no useful results [18]. This study aimed to determine the effect of glutamine supplementation on ileal histopathology in rats induced with EPEC for acute and chronic diarrhea.

This study aims to determine the relationship between glutamine supplementation and ileum histopathology in rats with acute and chronic diarrhea induced by EPEC.

Materials and Methods

This study was conducted in an experimental laboratory a randomized posttest-only control group design.

Experimental samples

The sample of this study consisted of male rats (*R. norvegicus* strain Wistar) aged 10–16 weeks, with a body weight of 160–250 g. Samples were divided into five groups with six rats per group: Negative control group (K), acute diarrhea group (A), acute diarrhea group supplemented with glutamine (Kyowa Hakko Bio Co., Made In Japan) at a dose of 810 mg/200g rat (B), chronic diarrhea group (C), and chronic diarrhea group supplemented with glutamine at a dose of 810 mg/200 g rat (D). The EPEC dose used was guided by several previous studies using EPEC as diarrhea induction and preliminary studies conducted by researchers. The dose of glutamine given to the rats was based on the human-to-animal dose conversion factors. The dose in humans is 45 mg/day at a body weight of 70 kg, converted into 0.018 mg/day for rat.

Experimental design

Rats are placed in plastic cages with a lid made of ram wire and are given a filter paper mat to observe rats' faces. The base is replaced 3 times a week. The cage is placed in a well-ventilated room with good air circulation. The environment of cage is made so that it is not humid, the temperature of the cage is maintained according to room temperature (20–26°C), and the exchange of dark and light every 12 h. Each rat is placed in its own cage. All rats are given food and drink is given *ad libitum* and monitored its health every day. Acute diarrhea was induced in Groups A and B using EPEC (2 ml with a population of 108 CFU/ml). Chronic diarrhea was induced in Groups C and D using EPEC (2 ml with a population of 108 CFU/ml, given doses of repetition every 3 days). Diarrheic rats in Groups A and C were given the standard feeding, while those in Groups B and D were given glutamine for 14 days at a dose of 810 mg/200 g/day as soon after diagnosed by acute and chronic diarrhea. On the 15th day after standard feeding and glutamine supplementation, glutamine is given at a dose of 810 mg/200 g which is dissolved with aqua as much as 4 ml orally using sonde every day for 14 days. The use of glutamine powder is a water-soluble substance. The rats were sacrificed

and samples from the ileal tissue were prepared. Ileum samples taken 15 mm toward the proximal from the caecum, taken along 10–15 mm terminal ileum. The ileum is cut to form sheet and clamped on thick plastic and fixed in 10% formalin for 24 h.

Histopathological analysis

Histopathological examination using an Olympus BX 51 made in 2013, zoom in for microscopic assessment at $\times 400$ magnification. Ileum tissue is inserted into the tissue cassette and a tissue process is carried out on the principle of dehydration, clearing, and paraffination. After that the blocking/embedding process is carried out with liquid paraffin at a temperature of 57–60°C, then carried out successively sectioning, staining with hematoxylin-eosin and mounting.

Statistical analysis

Statistical analysis was performed with SPSS version 19. The sample was tested to determine the effect of glutamine on the level of intestinal inflammation in the acute diarrhea group tested with the Chi-square test and the effect of glutamine in each group with Kruskal–Wallis test with $p < 0.05$ means significant.

Research ethics requirements

This study has obtained ethical approval from the Faculty of Medicine, University of Riau (15.3/UN19.5.1.1.8/UEPKK/2018).

Results

Ileum histopathology was assessed on day 15 after glutamine supplementation, in the K group (negative control), A (rats with acute diarrhea not given glutamine), B (rats with acute diarrhea given glutamine supplementation), C (rats with chronic diarrhea not given glutamine), and D (rats with chronic diarrhea given glutamine supplementation). Histopathological results were assessed to quantify the level of ileum inflammation with the spoiled Berthel scoring method and grouped into normal, minimal inflammation, mild inflammation, moderate inflammation, and severe inflammation (Table 1). The basis of this grouping is obtained from the research reference article.

Table 1: The effect of glutamine supplementation on the level of inflammation of rat ileum in acute diarrhea group

Group	Normal (%)	Minimal inflammation (%)	Mild inflammation (%)	Moderate inflammation (%)	Severe inflammation	p value
K	6 (100)	-	-	-	-	0.000
A	-	-	1 (16.7)	5 (83.3)	-	
B	-	1 (16.7)	4 (66.7)	1 (16.7)	-	

K=Control, A=Acute diarrhea, B=Acute diarrhea given glutamine.

The results obtained in group K did not show any inflammation (normal). While Group A, namely, rats with acute diarrhea, moderate inflammation occurred in a total of five rats (83.3%), and mild inflammation in one rat (16.7%). In Group B, namely, rats with acute diarrhea given glutamine supplementation, mild inflammation occurred in four rats (66.7%), and the remaining levels of inflammation occurred to at least one rat (16.7%). The Chi-square analysis showed that the inflammation level in acute diarrhea group was significant ($p < 0.05$).

There was a significant difference in the effect of glutamine supplementation in each group in acute diarrhea which was tested with Kruskal–Wallis test with a result of $p < 0.05$ (Table 2).

Table 2: The effect of glutamine supplementation on the level of inflammation of rat ileum in each group in acute diarrhea

Group	n	Average	p value
K	6	3.50	0.001
A	6	14.58	
B	6	10.42	

K=Control, A=Acute diarrhea, B=Acute diarrhea given glutamine.

In a chronic group also showed similar result, in Group C, moderate inflammation occurred in a total of four rats (80%), and mild inflammation in one rat (20%). While Group D, mild inflammation occurred in four rats (80%), and moderate inflammation occurred just in one rat (20%). An analytic test using the Chi-square test was performed with results displayed in Table 3.

Table 3: The effect of glutamine supplementation on the level of inflammation of rat ileum in chronic diarrhea group

Group	Normal (%)	Minimal inflammation (%)	Mild inflammation (%)	Moderate inflammation (%)	Severe inflammation	p value
K	6 (100)	-	-	-	-	0.000
C	-	-	1 (20)	4 (80)	-	
D	-	-	4 (80)	1 (20)	-	

K=Control, C=Chronic diarrhea, D=Chronic diarrhea given glutamine.

The analytical Kruskal–Wallis test results showed a $p < 0.05$, so it can be concluded that there were significant differences in the effect glutamine supplementation in each group in chronic diarrhea (Table 4).

Table 4: The effect of glutamine supplementation on the level of inflammation of rat ileum in each group in chronic diarrhea

Group	n	Average	p value
K	6	3.50	0.001
C	5	13.90	
D	5	9.10	

K=Control, C=Chronic diarrhea, D=Chronic diarrhea given glutamine.

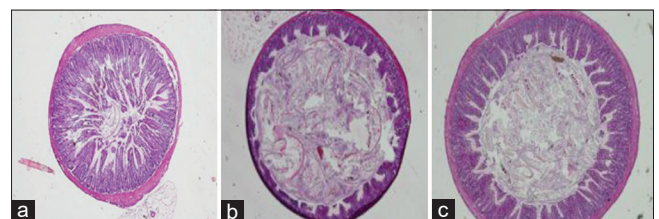


Figure 1: Cross-section of the ileum lumen in the acute diarrhea group. (a) Control, (b) Acute diarrhea, (c) Acute diarrhea+glutamine

Meanwhile, in Figure 1 and Figure 2, the intestinal lumen appears dilated with mucosal (M) thinning in Group A, while in Group B, there was an improvement in mucosal thickness and reduced intestinal distension compared to positive controls.

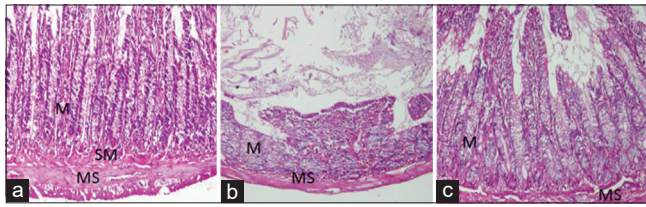


Figure 2: Sagittal section of the ileum lumen in the acute diarrhea group. (a) Control, (b) Acute diarrhea, (c) Acute diarrhea+glutamine

In Figure 3, Group A showed a decrease in the number of goblet cells accompanied by leukocyte infiltration in the lamina propria (LP), hyperemic blood vessels (H), and epithelial abnormalities such as erosion (E) or desquamation (D). In Group B, rats were given glutamine; an improvement in mucosal histology was found when compared with positive controls.

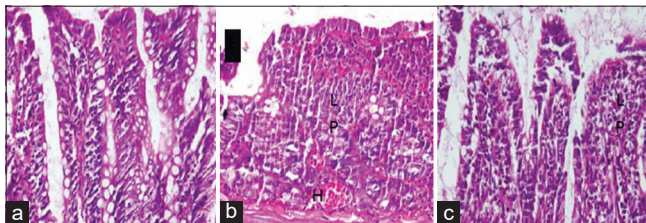


Figure 3: Goblet cell count, PMN infiltration in lamina propria and epithelial integration of the ileum lumen in the acute diarrhea group (a) control, (b) acute diarrhea, (c) acute diarrhea+glutamine

In Figure 4, Group A showed edema that appeared mainly in the submucosa (SM) of the small intestine with hyperemic (H) blood vessels, accompanied by leukocyte (L) infiltration in the LP. In Group B, there was an improvement in the intestinal mucosa and SM when compared with positive controls.

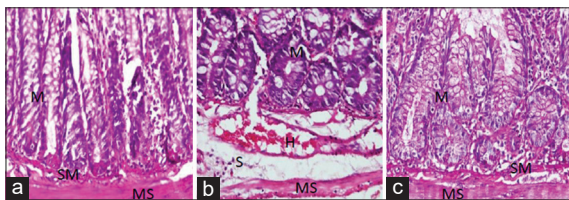


Figure 4: Edema of the ileum lumen in the acute diarrhea group (a) control, (b) acute diarrhea, (c) acute diarrhea+glutamine

There is distension of the intestinal lumen and thinning of the intestinal mucosa diarrhea due to EPEC and repair after glutamine.

Intestinal lumen was dilated with thinning mucosa (M) in Group C that had chronic diarrhea, in Group D who had chronic diarrhea given glutamine, there was an improvement in mucosal thickness and reduced intestinal distension when compared with positive control.

In Figure 5, Group A rats with acute diarrhea, there was a decrease in the number of goblet cells accompanied by leukocyte infiltration in LP, hyperemic blood vessels (H), and epithelial abnormalities in the form of erosion (E) or desquamation (D). In Group B rats given glutamine, there was an improvement in the histological picture of the intestinal mucosa when compared with positive controls. In Group C, rats with chronic diarrhea,

there was a decrease in the number of goblet cells accompanied by leukocyte infiltration in LP, hyperemic blood vessels (H), and epithelial abnormalities in the form of erosion (E) or desquamation (D), in Group D rats. Glutamine given an improved histological picture of the intestinal mucosa when compared with positive control.

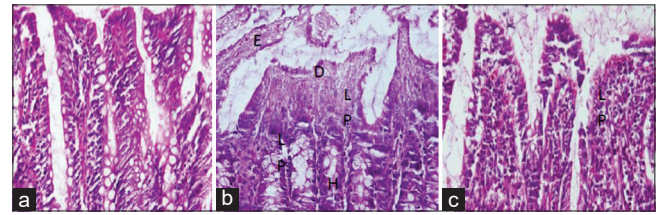


Figure 5: Goblet cell count, PMN infiltration in lamina propria and epithelial integration of the ileum lumen in the chronic diarrhea group (a) control, (b) chronic diarrhea, (c) chronic diarrhea+glutamine

In Figure 6, in Group A rats, edema was seen mainly in small intestinal SM with hyperemic blood vessels (H), accompanied by leukocyte infiltration (L) in the propria lamina. In Group B rats with glutamine administration, there was an improvement in the histological appearance of the intestinal mucosa and SM when compared with positive controls. In Group C rats, edema appeared mainly in the small intestinal SM with hyperemic blood vessels (H), accompanied by leukocyte infiltration (L) in the propria lamina. In Group D rats, glutamine administration showed improvement in the mucosal and SM histological features when compared with positive controls.

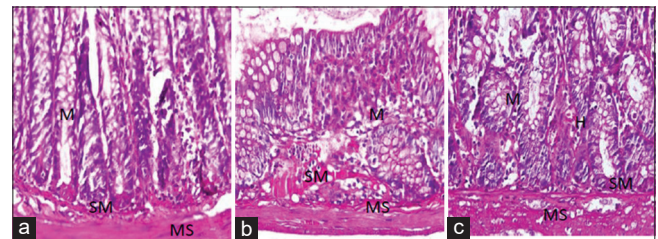


Figure 6: Edema of the ileum lumen in the chronic diarrhea group (a) control, (b) chronic diarrhea, (c) chronic diarrhea+glutamine

Discussion

In rats with diarrhea given glutamine supplementation, the level of inflammation is lower compared to rats with diarrhea that was not supplemented with glutamine. This proves that glutamine can reduce inflammatory reactions in the intestine, both in acute and chronic diarrhea. This result is supported by a study by Mariana *et al.* in 2017 which induced inflammation in the intestines of rats with 15 mg/kg cytarabine commonly used as a therapy for leukemia and lymphoma in humans, the rats were then treated with glutamine therapy at a dose of 150 mg/kg/day for 21 days and the administration of glutamine was found to reduce inflammation and increase the immune response in the intestine [19], [20].

In chronic group, these results fit with previous research done by Huang, where rats with chronic diarrhea given glutamine supplementation for 7 days found a significant improvement on villus high, surface of the villus, and expression of proliferating cell nuclear antigen as an index of the proliferation of cell, in comparison with the control group. Other studies conducted by Zhou Q, in the double-blinded, randomized, and controlled trial in adult patients who are experiencing chronic diarrhea in irritable bowel syndrome (IBS) given glutamine 5 g 3 times a day, showed improvement of intestinal permeability, rated from the increase in the ratio of lactose urine/mannitol, moreover also happens to repair of IBS scoring system, frequency of bowel movements, and frequency of peristaltic gut [21].

The results of histopathological examination in the acute and chronic diarrhea group obtained higher mean values in all categories of examination (SM edema, PMN infiltration in LP, reduced number of Goblet cells, and impaired epithelial integrity) compared to the group given glutamine. The histopathology in the group given glutamine was better than those without glutamine. The results of this study are in line with a study conducted by Chen *et al.* (1994) using Wistar rats induced with endotoxin, in which the group given glutamine showed improvements in the intestinal histopathology (improvement of mucosal thickness, villi height, crypt depth, and intestinal wall thickness) [22]. This improvement in the intestinal morphology is also in line with a study conducted by Tannuri *et al.* in 2000 using malnourished rats given glutamine supplementation which found morphological and morphometric improvements of the small intestine [23].

Furthermore, in intestinal cells of rats, oxidative stress injury due to reactive oxygen species (ROS) can trigger chain reactions of fat peroxidation and increase free radicals by oxidizing polyunsaturated fatty acids that cause oxidative cell damage, characterized by the production of malondialdehyde (MDA) as the final product of the fat peroxidation. In a 2014 study, Xu *et al.* found that a significant reduction in MDA occurred in rats given glutamine. This indicates that glutamine can protect rat intestines and repair tissue damage from cells mediated by ROS and intra-outer AOE. In addition, this study also found levels of tight junction proteins, including occludin, claudin, and cytoskeleton, that indicated improvement of the intestine [24].

EPEC infection begins with the attachment of pathogens to the intestinal mucosal surface, then invades and penetrates mucosal cells to stimulate pro-inflammatory cytokines which cause disruption of the absorption mechanism and damage to the tight junction, causing increased permeability, disruption of the sodium-potassium pump, inhibition of water and electrolyte absorption, and fluid accumulation in the intestine that ends with diarrhea and damage to the histopathology of the small intestine [7], [25].

Glutamine has been known to be the main source of energy for the development and differentiation of intestinal epithelial cells. Enteral administration of glutamine stimulates protein synthesis in the intestinal mucosa, protects enterocytes from apoptosis, and activates immune cells. This causes glutamine to maintain and repair damaged intestinal cells [15], [26]. However, in the case of damaged intestinal cells, inadequate levels of glutamine can cause cells to be unable to repair effectively; therefore, glutamine supplementation is needed [24].

Conclusion

This study concluded that supplementation of glutamine will reduce the level of inflammation and there is a histopathological improvement of the ileum in rats with acute and chronic diarrhea. The results of this study can be the beginning of further research on the effect of glutamine on diarrhea patients.

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