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Effects of Caloric Intake on Intestinal Mucosal Morphology and Immune Cells in Rats Treated with 5-Fluorouracil

Mariko Murakami, Norifumi Sato*, Katsufumi Tashiro, Tsuyoshi Nakamura, and Hiroaki Masunaga

EN Otsuka Pharmaceutical Co., Ltd., R&D Laboratories, 4-3-5, Nimaibashi, Hanamaki, Iwate 025-0312, Japan

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Summary **Anticancer drugs have been reported to damage the intestinal mucosa. We evaluated the effects of caloric intake on the mucosal morphology and immune cells in rats treated with 5-fluorouracil (5-FU). Rats were received a liquid diet plus 5-FU treatment for 8 days as follows: Low calorie group (25 kcal/day with 5-FU), Normal calorie group (50 kcal/day with 5-FU), and Control group (50 kcal/day with saline). The mucosal morphology, cell numbers and phenotypes of spleen and intraepithelial lymphocytes (IEL) were assessed. As compared with the control group, the villus heights were significantly lower in the Low calorie group, but not significantly lower in the Normal calorie group. The total cell yield from the spleen, CD4+ and CD8+ T cells decreased in the Low and Normal calorie group, but these changes were less pronounced in Normal calorie group. The total cell yield from the IEL also decreased in the Low calorie group, but not in the Normal calorie group. Our study demonstrated that sufficient caloric intake attenuated the damages in intestinal morphology and in the immune cell numbers. Clinically, nutritional support would be expected to be one approach to reducing the risk of bacterial translocation or infection induced by chemotherapy.**

Key Words**: 5-FU, mucosal atrophy, nutritional support**

Introduction

Anticancer drugs have side effects such as gastrointestinal injury, leucopenia, stomatitis and diarrhea, which sometimes limit the chemotherapy, and influence the quality of life of these patients [*1*, *2*].

5-Fluorouracil (5-FU) is widely used on solid tumors [*3*]. It has been reported that the inhibition of DNA synthesis caused by 5-FU injures the gastrointestinal tract mucosa [*4*], and causes changes in intestinal mucosal morphology [*5*]. 5-FU also injures immune cells of the host, thus causing immunosuppression [*6*, *7*]. These changes in mucosal morphology and immune function might result in bacterial translocation [*5*].

Many cancer patients are subjected to a reduced dietary intake because of tumor-induced alterations in metabolism, and/or treatment such as chemotherapy and radiation therapy. Despite many advances in nutritional therapy, the incidence of malnutrition remains high [*8*]. Reduced energy intake causes the changes in mucosal morphology and gut barrier function [*9*], or immune cell function [*10–13*]. Therefore, it appears that the reduced caloric intake could deteriorate the 5-FU induced gastrointestinal injury. In this study, we evaluated the effects of nutritional support on 5-FU induced intestinal injury and immunosuppression. We compared the effects of low and normal caloric intake on mucosal morphology and immune cell numbers in rats treated with 5-FU.

*To whom correspondence should be addressed.
Tel: +81-198-30-1883 Fax: +81-198-30-1819
E-mail: n-sato@enotsuka.co.jp

Materials and Methods

Animals

Male, 5 week-old Sprague-Dawley rats (CLEA Japan, Inc., Tokyo) were housed in wire cages under controlled temperature (23 ± 3°C) and humidity conditions (50 ± 20%), with a 12-h light-dark cycle. The rats were fed commercial laboratory chow, and were allowed drinking water *ad libitum* for about one week before the surgery. The studies reported herein conform to the guidelines for the care and use of laboratory animals established by the Animal Use and Care Committee of EN Otsuka Pharmaceutical Co., Ltd.

Surgical procedure for gastrostomy

After an overnight fast, the rats were anesthetized with pentobarbital (45 mg/kg body weight, i.p.). A laparotomy was performed through a midline incision. A catheter (5 Fr size umbilical catheter, Nippon Sherwood Medical Ind., Tokyo) was then inserted into the stomach. The distal end of the catheter was tunneled subcutaneously through the left lateral abdominal wall, and exited at the interscapular region. The catheter was attached to a swivel spring, which allowed the rats freedom of movement in their individual cages. After an overnight fast, the rats were fed chow and were allowed drinking water *ad libitum*.

Experimental design

The experimental groups and design are outlined in Table 1 and Fig. 1. The day before starting 5-FU treatment, the rats were randomized into the three groups: Low calorie group (n = 9) as a malnutrition model, Normal calorie group (n = 10) as a nutritional support model and Control group (n = 10). The rats received 25 kcal/day (Low calorie group), or 50 kcal/day (Normal calorie and Control groups) of liquid diet. The calorie administration of 25 kcal/day of liquid diet was based on the amount that the rats fed *ad libitum* during the 5-FU treatment (data not shown). The composition of the liquid diet (Racol®, EN Otsuka Pharmaceutical Co., Ltd, Iwate) is shown in Table 2. The liquid diet was given by an infusion pump for 16 h. The rats in the Low calorie and Normal calorie groups were treated with 5-FU (20 mg/kg, i.p.), and the rats in the Control group were treated with an equal volume of saline for 8 consecutive days. On day 8, rats were sacrificed, and the spleen was excised for spleen cell isolation from five rats in each group. The intestinal mucosal morphology was also observed in the same rats. The small intestine was excised for intraepithelial lymphocyte (IEL) isolation from the remaining rats.

Intestinal mucosal morphology

Duodenum samples were taken at 5 cm before the ligament of Treitz, and jejunum samples were taken at 10 cm after the ligament of Treitz. The wet weight of intestinal samples were weighed, and expressed as mg/cm. The proximal 2 cm segment was cut off, and opened longitudinally along the mesenteric side. The serosal side of the intestine was placed on paper for orientation, and then inserted into a 10% buffered formalin solution. The samples were embedded in paraffin, cut 5 μm thick, and strained with hematoxylin and eosin. The villus heights of the duodenum and jejunum were measured using an ocular micrometer under light microscopy. Ten visual fields from each specimen were measured, and the average value was taken.

Table 1. Experimental groups

Groups	Administration of liquid diet	Injection of 5-FU (20 mg/kg/day)
Control	50 kcal/day	−
Low calorie	25 kcal/day	+
Normal calorie	50 kcal/day	+

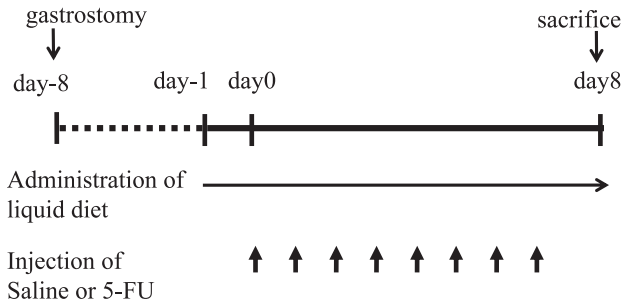

Fig. 1. Experimental design. After a gastrostomy, rats were received a liquid diet plus 5-FU treatment for 8 days as follows: Low calorie group (25 kcal/day with 5-FU treatment), Normal calorie group (50 kcal/day with 5-FU treatment), and Control group (50 kcal/day with saline). The calorie administration of 25 kcal/day of liquid diet was based on the amount that the rats fed *ad libitum* during the 5-FU treatment (data not shown). The liquid diet was given by an infusion pump for 16 h.

Cell isolation

The spleen and small intestine were placed in tissue culture medium RPMI 1640 (Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 g/liter streptomycin, and 100,000 IU/liter penicillin. The spleen was then passed through a sterile wire mesh to remove any tissue debris. After centrifugation, the pellets were incubated with 0.83% NH_4Cl Tris buffer for 10 min to lyse the red blood cells. The cells were washed twice, and resuspended in tissue culture media.

The IEL were isolated by using the method reported previously [*14*]. After removing the small intestine, it was placed in Hank's balanced salt solution (HBSS, Gibco,

Table 2. Composition of liquid diet (per 100 kcal, 100 mL)

Ingredient	Contents	Unit
Protein	4.38	g
Fat	2.23	g
Carbohydrate	15.62	g
Minerals		
Sodium	73.8	mg
Potassium	138	mg
Calcium	44	mg
Magnesium	19.3	mg
Phosphorus	44	mg
Chloride	117	mg
Iron	625	μg
Zinc	640	μg
Manganese	133	μg
Copper	125	μg
Vitamins		
Retinol palmitate	207	IU
Cholecalciferol	13.6	IU
Tocopherol acetate	650	μg
Phytonadione	62.5	μg
Thiamine	380	μg
Riboflavin	245	μg
Pyridoxine	375	μg
Cyanocobalamin	0.32	μg
Ascorbic acid	25	mg
Nicotinamide	2.5	mg
Pantotheic acid	958	μg
Folic acid	37.5	μg
Biotin	3.86	μg

GrandIsland, NY.) containing 5% FBS. The intestine was turned inside out, cut into 2 cm pieces and incubated with HBSS (Ca, Mg-free, 5% FBS, 5 mM EDTA) for 45 min at 37°C in a water shaker (150 rpm). The supernatants were then filtered through a glass wool column. After centrifugation, the pellets were suspended in 44% Percoll (Amersham Bioscience AB, Uppsala, Sweden), and the cell suspensions were overlaid onto 70% Percoll. After centrifugation for 20 min at 600 g at 20°C, the lymphocytes were recovered from the 44/70% interface and washed in tissue culture media. The lymphocytes were finally resuspended in tissue culture medium as described above.

Flow cytometry

Fluorescein isothiocyanate (FITC)-conjugated anti-rat CD4, CD8, TCRαβ and TCRγδ antibodies were obtained from BioLegend (San Diego, CA). CD4 and CD8 were used as general markers of T cell subsets. TCRαβ and TCRγδ were used to identify the αβ chain T-cell receptor and the γδ chain T-cell receptor of IEL, respectively. To determine the phenotypes of the lymphocyte, spleen cells and IEL (2×10^5 cells/100 μL) were incubated with monoclonal antibodies (10 μL) for 30 min in the dark on ice. The cells were washed twice with ice-cold PBS/1% FBS, and the antigen expressions were then analyzed with a flow cytometer FC500 (Coulter, Miami, FL).

Statistical analysis

The results were expressed as means ± SD. The statistical analysis was performed by Tukey-Kramer's HSD test and p values less than 0.05 were considered to be statistically significant.

Results

Body weight

Before 5-FU treatment (On day-1), there were no significant differences in body weight among the three groups (Control group: 224.7 ± 13.2 g, Low calorie group: 228.6 ± 10.0 g, and Normal calorie group: 227.0 ± 11.0 g). On day 8, body weight of the Low calorie group (182.7 ± 9.8 g) was lower than that of the Control and Normal calorie groups (231.4 ± 10.8 and 231.1 ± 8.2 g, respectively). In the Control and Normal calorie groups, the body weight was maintained during the experimental period, and there was no significant difference in body weight between these two groups (Fig. 2).

Intestinal mucosal morphology

The wet weights of both the duodenum and jejunum in the Low calorie group were lower than those of the Control group, but there were no statistically significant differences. The wet weight of the duodenum in the Normal calorie group was higher than that of the Low calorie group. The villus heights in both the duodenum and jejunum in the Low calorie group were lower than in the Control group (Table 2 and Fig. 3). There were no significant differences in villus height between the Control and Normal calorie groups. All rats in the three groups didn't have diarrhea during the experimental period.

Total cell yield

The number of cells isolated from the spleen and IEL were significantly decreased in the Low calorie group as compared with the Control group (Fig. 4). In the Normal calorie group, the number of spleen cells was significantly decreased as compared with the Control group, but significantly higher than the Low calorie group. There was no significant difference in the number of IEL between the Control and Normal calorie group.

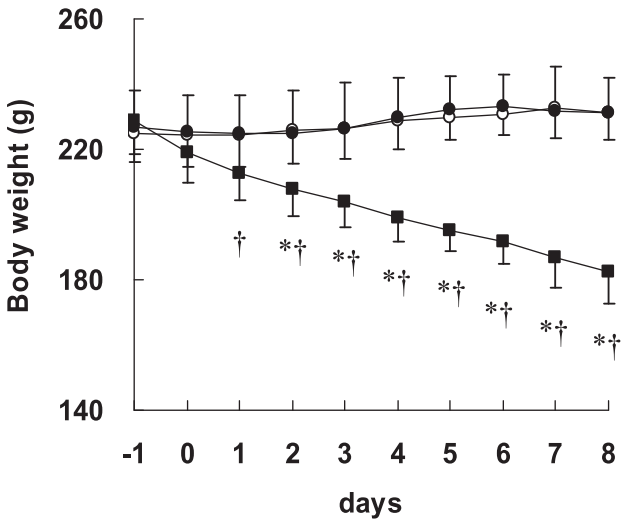

Fig. 2. Effect of caloric intake on the body weight of 5-FU treated rats. The data represent the body weight of Control group (open circles; $n = 10$), Low calorie group (closed squares; $n = 9$) and Normal calorie group (closed circles; $n = 10$). After a gastrostomy, rats were received a liquid diet plus 5-FU treatment for 8 days as follows: Low calorie group (25 kcal/day with 5-FU treatment), Normal calorie group (50 kcal/day with 5-FU treatment), and Control group (50 kcal/day with saline). Significant differences between Low calorie group and Normal calorie group were observed after day 1. Values are means ± SD. *$p<0.05$ represents statistical differences compared with Control group; †$p<0.05$ represents statistical differences compared with the Normal calorie group; Data were analyzed by using Tukey-Kramer's HSD test.

Lymphocyte phenotypes

The percentage of CD4+ T cells in the spleen in the Low calorie group was significantly higher than in the Control group (Table 3). There were no significant differences in the percentages of CD8+ T cell in the spleen and IEL phenotypes between the Low calorie group and the Control group. There were no significant differences in lymphocyte phenotypes in the spleen and IEL between the Control and Normal calorie groups.

The absolute numbers of CD4+ and CD8+ T cells in the spleen in both Low calorie group and Normal calorie groups were significantly decreased as compared with the Control group (Table 4). In the Normal calorie group, the absolute numbers of CD4+ and CD8+ T cells in the spleen were significantly increased as compared with the Low calorie group. The absolute numbers of IEL phenotypes in the Normal calorie group was closer to the numbers from the Control group than the Low calorie group, whereas the numbers from the Low group tended to belower than the Normal and Control groups.

Discussion

The present study demonstrated the effects of different levels of caloric intake on the mucosal morphology and immune cells in rats treated with 5-FU. Although 5-FU is known to influence mucosal morphology and immune function, the effects of the caloric intake on these changes has not been previously well characterized. Our data indicate that the combination of reduced energy intake and 5-FU treatment results in significant atrophy of the mucosa and changes in systemic and mucosal immunity. These changes can be minimized or eliminated when sufficient nutrients are administered enterally.

The intestinal mucosa is the barrier created by the physical structure of the epithelium. In addition, the gut-associated

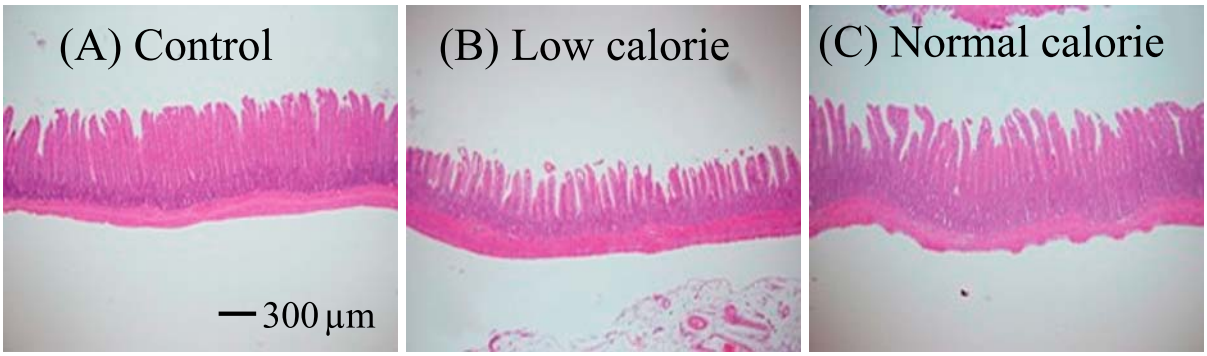

Fig. 3. Structure of jejunum. (A)Control group, (B) Low calorie group, and (C) Normal calorie group. Rats were received a liquid diet plus 5-FU or saline treatment from day 0 to day 7. On day 8, rats were sacrificed. The duodenum and jejunum tissues were removed from five rats per group. Each sample was processed for hematoxylin and eosin staining as described in Material and methods.

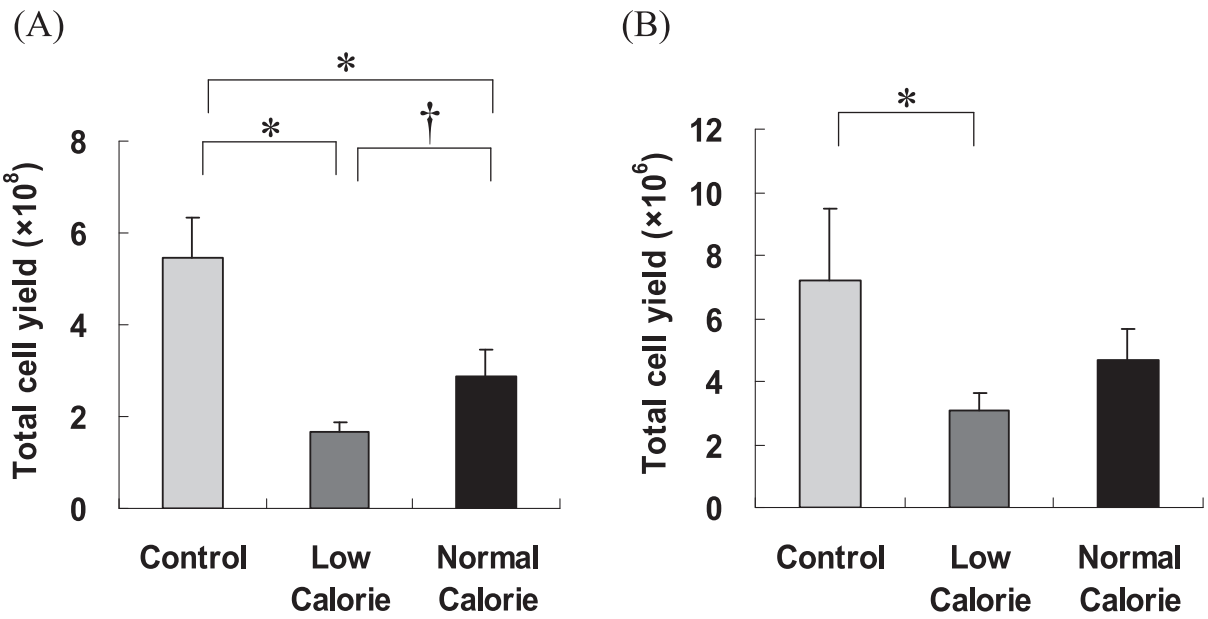

Fig. 4. Effect of caloric intake on total cell yield from spleen (A) and intraepithelial lymphocytes (B). Rats were received a liquid diet plus 5-FU or saline treatment from day 0 to day 7. On day 8, rats were sacrificed. The spleen cells were isolate from five rats per group and intraepithelial lymphocytes were isolated from five rats (Normal calorie and Control group) or four rats (Low calorie group). The 5-FU induced low caloric intake decreased the immune cell numbers. Values are means ± SD. *$p<0.05$ represents statistical differences compared with Control group; †$p<0.05$ represents statistical differences compared with the Normal calorie group; Data were analyzed by using Tukey-Kramer's HSD test.

Table 3. Effects of caloric intake on the intestinal mucosal morphology.

Groups	duodenum		jejunum	
	Wet weight (mg/cm)	Villus height (μm)	Wet weight (mg/cm)	Villus height (μm)
Control	112.4 ± 25.4	466 ± 36	83.1 ± 19.1	519 ± 52
Low calorie	84.8 ± 6.3†	362 ± 38*	70.1 ± 11.4	339 ± 72*
Normal calorie	116.4 ± 17.3	407 ± 88	91.7 ± 28.3	420 ± 118

The 5-FU induced low caloric intake decreased the villus height in duodenum and jejunum. Sufficient caloric intake attenuated these changes in intestinal morphology. Values are means ± SD. *$p<0.05$ represents statistical differences compared with Control group; †$p<0.05$ represents statistical differences compared with the Normal calorie group; Data were analyzed by using Tukey-Kramer's HSD test.

Table 4. Changes in the absolute numbers of lymphocyte subsets in the spleen and IEL.

Groups	Spleen ($\times 10^7$)		IEL ($\times 10^5$)			
	CD4+	CD8+	CD4+	CD8+	TCRαβ+	TCRγδ+
Control	13.0 ± 1.7	4.7 ± 0.9	2.2 ± 1.2	20.4 ± 12.8	18.3 ± 8.8	3.1 ± 2.1
Low calorie	5.3 ± 0.9*†	1.3 ± 0.5*†	0.9 ± 0.6	8.2 ± 4.1	9.4 ± 4.7	1.2 ± 0.4
Normal calorie	8.3 ± 1.4*	2.7 ± 0.6*	1.9 ± 0.8	21.2 ± 11.4	18.4 ± 9.5	3.3 ± 1.8

Spleen cells and intraepithelial lymphocytes (IEL) were isolated on day 8, and the antigen expressions were then analyzed with a flow cytometer. The 5-FU induced low caloric intake decreased the CD4+ and CD8+ cell numbers in spleen. Values are means ± SD.*$p<0.05$ represents statistical differences compared with Control group; †$p<0.05$ represents statistical differences compared with the Normal calorie group; Data were analyzed by using Tukey-Kramer's HSD test.

lymphoid tissue (GALT) is an immunologic barrier in the intestine. Bacterial translocation is promoted by: (i) physical disruption of the mucosal barrier, (ii) intestinal bacterial overgrowth, or (iii) suppression of the host immune defenses [*15*]. Under stressed conditions, indigenous bacteria can pass through the epithelial cells, enter the lamina propria, and appear in the mesenteric lymph nodes (MLN). From the MLN, these bacteria may spread to other sites, such as the liver, spleen, kidneys, and blood [*16*]. Bacterial translocation causes sepsis-like manifestations after cancer chemotherapy [*5*]. Therefore, preventing bacterial translocation during chemotherapy is important for cancer patients.

In the current study, the decreases in the villus height correlated with the magnitude of caloric intake with 5-FU treatment. In the clinical field, diarrhea is one of the important side effects of 5-FU. It has been reported that the degree of diarrhea was well correlated with the histological changes of the gastrointestinal mucosa [*5*]. In our study, we observed that all rats treated with 5-FU didn't have diarrhea, although mucosal damage was caused by 5-FU. We administered 5-FU at 20 mg/kg for rats, that dose might be relatively small dose to examine the effect to diarrhea. Under this condition, it is also possible that the changes in the mucosal morphology might be due to a loss of nutritional substrates and hormonal stimulation [*17–19*]. With respect to the mechanisms of the loss of villus length, dietary restriction reportedly results in decreased epithelial cell proliferation and increased apoptosis [*9*]. The maintenance of gut mucosal homeostasis depends on a balance between cell proliferation and cell death. Anticancer drugs might increase mucosal epithelial cell apoptosis because of the rapid turnover of the cells. Therefore, chemotherapy and reduced energy intake might worsen the overall mucosal morphology.

Reduced energy intake and 5-FU treatment also caused a loss of the total cell yield in the spleen and IEL, suggesting an impairment of the immunologic barrier. The absolute numbers of CD4+ and CD8+ T cells in the spleen were preserved by sufficient caloric intake. CD4+ and CD8+ T cells in the IEL tended to be preserved by sufficient caloric intake, but, there were no statistically significant differences. IEL is one of the major effector components in the GALT, and produces various cytokines such as interferon (IFN)-γ, IL-10, transforming growth factor-beta (TGF-β), and regulates the immune response to foreign antigens. Any depletion of the CD4+ and CD8+ T cells within the GALT could increase bacterial translocation [*15*, *20*].

In clinical practice, parenteral nutrition (PN) is often chosen for nutritional support. However, PN induces mucosal villus atrophy, epithelial cell apoptosis, and bacterial translocation in the mouse model [*21*, *22*]. Sun *et al.* reported that the lack of enteral nutrition, rather than absolute caloric levels, is responsible for many of the adverse effects of PN [*22*]. They reported that PN-associated epithelial derangements were worsened by reducing the caloric PN delivery, and even with full caloric delivery, the mucosal morphology and function are compromised in comparison to the control state (enteral nutrition). Therefore, PN during the chemotherapy might lead to more adverse changes of the mucosal morphology and gut barrier function. PN administration also results in phenotypic and functional changes in the GALT [*23*, *24*]. PN feeding in mice resulted in atrophy of the GALT and T-cell and B-cell populations, and decreased in intestinal IgA levels [*24*]. Cytokine mRNA levels in the IEL were also decreased by PN [*23*]. These mucosal immune changes might account for the elevation in bacterial translocation. Enteral nutrition during chemotherapy would be effective to maintain the mucosal barrier function.

The patient's nutritional status affects the toxicity of chemotherapy. Host toxicity such as leucopenia and diarrhea caused by 5-FU was increased by protein energy malnutrition [*25*]. With respect to the mechanism responsible for this phenomenon, the metabolism rate of 5-FU is implicated [*26*]. Protein depletion results in a decreased rate of hepatic metabolism and clearance of 5-FU, and decreased activity of hepatic dihydropyrimidine dehydrogenase (DPD) which deactivates 5-FU [*27*]. Thus, nutritional support is important for tissue regeneration and drug metabolism.

Recently, certain nutrients have been shown to be beneficial during cancer chemotherapy. In our present study, we used the enteral nutrition enriched with soybean as a protein source and omega-3 fatty acids as a fat source. Mitsugi *et al.* reported the protective effect of the enteral diet containing soybean protein and omega-3 fatty acid from severe diarrhea and death caused by methotrexate [*28*]. Soybean containing diet has been shown to protect animals from methotrexate-induced anorexia and diarrhea when compared with casein [*29*]. Moreover, omega-3 fatty acid has been shown to reduce the gastrointestinal, hematological or cardiac side effects of various chemotherapeutic treatments [*30*, *31*]. Thus, the supplementation of these nutrients during nutritional support could contribute to attenuate the mucosal injury as well as caloric intake. Further studies are needed on the effect of these nutrients on the mucosal injury by 5-FU chemotherapy.

In summary, reduced energy intake during 5-FU treatment resulted in significant atrophy of the intestinal mucosa, and a decrease in the numbers of systemic and mucosal immune cells. Preservation of the mucosal morphology and immune cells with sufficient energy intake might result in an increased resistance to infection. Clinically, nutritional support during chemotherapy could be one approach to reducing the risk of anticancer drugs induced bacterial translocation or infection.

Abbreviations

5-fluorouracil, 5-FU; intraepithelial lymphocytes, IEL; fetal bovine serum , FBS; Hank's balanced salt solution, HBSS; Fluorescein isothiocyanate , FITC; parenteral nutrition, PN; interferon, IFN; transforming growth factor-beta, TGF-β; dihydropyrimidine dehydrogenase, DPD.

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