The effects of oral nutritional formula enriched with arginine, omega-3 fatty acids and nucleotides on methotrexate-induced experimental intestinal mucositis

Elvan Yilmaz Akyuz¹, Cebrail Akyuz², Gul Kiziltan³, Ahmet Ozer Sehirli⁴, Sule Cetinel⁵
¹ University of Health Sciences, Faculty of Health Sciences, Department of Nutrition and Dietetics, Istanbul/Turkey - E-mail: yilmaz.elvan@gmail.com; ²Haydarpasa Numune Education and Research Hospital, Department of Surgery, Istanbul/Turkey; ³Baskent University, Faculty of Health Sciences, Department of Nutrition and Dietetics, Ankara/Turkey; ⁴ Near East University, Faculty of Dentistry, Department of Pharmacology, Lefkosa/Cyprus; ⁵ Marmara University, Faculty of Medicine, Department of Histology and Embryology, Istanbul/Turkey

Summary. The aim of this study to investigate the effect of an immunonutritional (IN) oral formula enriched with arginine, omega-3 fatty acids and nucleotides in methotrexate (MTX)-induced experimental intestinal mucositis model. In the study, 32 rats were divided into four groups consisting eight animals in each. Control group fed for 5 days with only saline with gavage, IN group fed for 5 days with an oral IN formula three times a day, MTX group with an intraperitoneal single dose MTX (20 mg/kg), followed by saline with gavage, MTX-IN group with a single dose of MTX, followed by an oral IN formula three times a day. The blood and jejunal tissue sample were collected and then the rats were sacrificed on the sixth day of the study. The level of TNF-α, IL-1β in serum, luminol, lusigenin, glutathione, myeloperoxidase, malondialdehyde and Na-K+ATPase in the jejunal tissue samples were analyzed. Histopathological examination was performed in the jejunal tissue samples. In the MTX group, TNF-α, IL-1β levels in serum, and luminol, lusigenin, malondialdehyde and Na-K-ATPase in the jejunal tissue samples were analyzed. Histopathological examination was performed in the jejunal tissue samples. In the MTX group, TNF-α, IL-1β levels in serum, and luminol, lusigenin, malondialdehyde levels, and myeloperoxidase activity in tissue samples were found significantly higher than the control group. Glutathione and Na-K-ATPase levels were lower in the jejunal tissue of the MTX group compared to control group. However, the supplementation of IN with the MTX resulted in a significant increase in glutathione and Na-K-ATPase levels. Severe epithelium loss and inflammatory cell increase were observed in the MTX group on histological examination, whereas these parameters were regressed in the MTX-IN group. Increasing in the mitosis rate of enterocytes and inflammatory cell density decreased with the IN. In conclusion, this study shows that chemotherapy has adverse effects on intestinal mucosa and the IN formula has a protective effect of on MTX-associated intestinal damage.

Key words: methotrexate, intestinal mucositis, immunonutrition

Abbreviations

MTX; methotrexate
IN; immunonutrition
TNF-α; tumor necrosis factor-alpha
IL; interleukin
MDA; malondialdehyde
MPO; myeloperoxidase
GSH; glutathione
ELISA; enzyme immunoassay
FOR; free oxygen radicals

RLU; relative light units
AUC; area under the curve
H&E; hematoxylin-eosin

Introduction

Chemotherapy-induced gastrointestinal mucositis is significantly dose-limiting in cancer treatment and has severe side effects (1). Gastrointestinal mu-
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Mucositis develops in 15 to 40% of the cases after standard dose chemotherapy and in 76 to 100% of the cases after high dose chemotherapy (2). Clinically, it is accompanied by oral symptoms such as oral pain, erythema, ulceration, and gastrointestinal symptoms such as swelling, abdominal pain and diarrhea (3, 4). Mucositis increases the duration of hospital stay, hospital costs, narcotic use for the pain, the need for parenteral feeding, and negatively impact the quality of life (5).

Based on the approach developed and accepted within the past two decades, apart from the standard nutrition, the form of nutrition enriched with various nutritional elements is used to enhance the immunity of the patient. Many studies have shown that supporting the immunity can control the duration and intensity of acute phase inflammatory response in certain patients (6-8). Nutritional elements such as arginine, glutamine, dietary nucleotides, polyunsaturated fatty acids, antioxidants, copper, selenium, and zinc are known to play a key role in the steps within the complex structure of the inflammatory response (9). Several studies have shown that immunonutrition (IN) improves immune responses, controls inflammatory changes, modulates the synthesis of acute phase proteins, increases intestinal oxygenation and barrier function after injury; furthermore, it also reduces septic morbidity and mortality (10, 11).

In this experimental study, we aimed to investigate the effect of an oral IN formula enriched with arginine, omega-3 fatty acids, and nucleotides on intestinal mucositis, which is one of the side effects of methotrexate (MTX), and which has been known to have toxic effects on several systems.

Materials and methods

Ethics and animals

The experimental groups consisted of 32 Wistar-Albino rats purchased from a University, Experimental Animals Practice and Research Center. The study was performed after obtaining ethical committee approval from a Marmara University Local Ethical Committee of Animal Experiments (protocol code: 76.2012. mar). The rats were fed using standard pellet feed, and were given free access to water during the experiment.

All rats were kept in polypropylene cages between 22-24°C and under standard conditions concordant with the 12-hour night-day cycle.

Experimental design

The rats were divided into four groups with eight animals in each, and were fed for 5 days. Control group: only saline with gavage, IN group: an oral IN formula (5 mg/kg) three times a day, MTX group: intraperitoneal single dose MTX (20 mg/kg), followed by saline with gavage, MTX-IN group: a single dose of MTX, followed by an oral IN formula (5 mg/kg) three times a day.

The rats were fed with standard feed and water in every stage of the experiment. All groups underwent laparotomy on day 6. Blood samples were collected to evaluate tumor necrosis factor-alpha (TNF-α) and interleukin (IL)-1β levels, and samples were collected to evaluate oxidative damage and mucositis in the intestinal tissue. Some of the samples were kept at -80°C for the measurement of malondialdehyde (MDA), myeloperoxidase (MPO), glutathione (GSH), Na+-K+ ATPase, luminol, lucigenin, whereas some of the samples were fixed in 10% formaldehyde for histopathological analysis.

Immune supplementation therapy

The rats in the IN and MTX-IN groups received an immune supplement containing arginine, ω-3 fatty acids, nucleotides and vitamins. The enteral formula contains 237 ml 334 kcal, 18.1 g proteins (4.2 g L-arginine), 44.8 g carbohydrates, 9.2 g lipids (1.4 g ω-3), 3.3 g fiber, 0.43 g nucleotides, vitamins and minerals. It was served for five days prior to surgery, with a daily dosage of three 5 mg/kg oral.

Serum TNF-α and IL-1β measurement

Serum TNF-α measurement was performed using Enzyme Immunoassay (ELISA) method with Biosource kit (ELISA, Biosource Europe S.A. Catalog No. KRC 3014, Nivelles, Belgium) and Elx808IU Ultra Microplate device. In addition, IL-1β measurement was performed using enzyme immunoassay method with Biosource kit (ELISA, BioSource Catalog No. KRC 0011, Nivelles, Belgium) and Elx808IU Ultra Microplate device.
**MDA measurement**

Measurement of MDA, one of the products of lipid peroxidation, was performed according to the method described by Ohkawa et al. (12). Reaction product of MDA and thiobarbituric acid in acidic environment was observed spectrophotometrically under 532 nm and the results were presented as nmol/g tissue.

**MPO activity measurement**

Tissue MPO activity was measured using the procedure described by Hillegass et al. (13). The samples of 0.2 to 0.3 g collected from the tissues were diluted 10x using 20 mM K2HPO4 (pH=7.4) and homogenized, and then centrifuged at 12,000 rpm for 10 min at 4°C. Samples were homogenized using 50 Mm K2HPO4, which includes an equivalent volume of 0.5% hexadecyltrimethylammonium hydroxide, and the MPO activity was measured by spectrophotometric measurement of the H2O2-dependent oxidation of O-dianisidine 2HCl. One unit of enzyme activity was defined as the change in 460 nm absorbance (1.0 ml/min) at 37°C, and MPO activity was expressed in U/g tissue.

**GSH measurement**

The GSH activity was measured using the modification of the Ellman procedure (14). After the homogenates were centrifuged at 3,000 rpm for 10 min at 4°C, 0.5 mL supernatant was collected and 2 ml of 0.3 M Na2HPO4, 2H2O solution was added on top of the supernatant. Then, 0.2 mL dithiobisnitrobenzoate (0.4 mg/mL 1% sodium citrate) solution was added to the mixture. After the mixture was shaken, it was incubated at room temperature for five minutes, and spectrophotometric measurement was performed at a wavelength of 412 nm. The GSH levels were calculated using 13,600 moles-1 cm-1 value. The results were expressed in GSH/g tissue.

**Measurement of Na+-K+ATPase levels**

The Na+-K+ATPase activity in the supernatant was found by spectrophotometrically measuring the inorganic phosphate comprising 3 mM adenosine triphosphate added to the environment during the course of incubation at a wavelength of 690 nm. Enzyme activity was expressed in nmol Pi mg-1 protein h-1. Protein concentration of the supernatant was identified by Lowry method (15).

**Detection of Free Oxygen Radicals (FOR) in the tissue by the chemiluminescence method**

The measurement of FOR in the tissue samples was performed using the chemiluminescence method. Luminol (5- amino-2,3-dihydro-1,4 phtalazinedione, 0.2mM) or lucigenin (bis-N methylacridinium nitrate, 0.2mM) intermediate was added to cell suspensions. Lucigenin is sensitive to superoxide radical, whereas luminol is sensitive to hydroxyl anion, hydrogen peroxide, hypochloride and hydroperoxyl radicals. The tubes with luminol and lucigenin were counted under luminometer (Berthold EG & G Minilumat LB 9506, Germany) in 1-min intervals for 10 minutes and the results were expressed in the Relative Light Units (RLU) using the Area Under the Curve (AUC) (16).

**Histological analysis**

Tissue samples collected from the rats were kept in 10% formaldehyde solution and after 24 hours of detection, routine histological tissue follow-up was performed and the samples were placed in paraffin blocks, and 4-5 μm sections were prepared using microtome. Obtained tissue sections were dyed with hematoxylin-eosin (H&E) and standard protocol was performed. Prepared samples were visualized under light microscope (Olympus CX 41).

This was a single blind study, performed by a histopathologist who was blinded to the groups. Mucosal damage in the intestinal tissue samples was evaluated histopathologically; inflammation intensity, epithelial structure, mitosis and goblet cells were analyzed morphologically.

**Statistical analysis**

Statistical analysis was performed using GraphPad Prism 3.0 for Windows (GraphPad Software, San Diego, CA, USA). Descriptive data were expressed in mean ± standard error (mean ± SE). The Kolmogorov-Smirnov test was used to analyze the normally distributed variables. The variables were compared using analysis of variance (ANOVA) and Tukey’s multiple comparison tests. A p value of <0.05 was considered statistically significant.
Results

No significant differences in parameters were detected between the control and IN groups ($p>0.05$). Thus, MTX and MTX+IN groups were compared with the control group.

As shown in Table 1, plasma levels of the pro-inflammatory cytokines (TNF-$\alpha$ and IL-1$\beta$) in the MTX group was significantly higher than the control group ($p<0.001$), and IN treatment substantially decreased the elevation of plasma levels of these cytokines ($p<0.001$).

In the group treated with MTX, cellular antioxidant GSH levels decreased ($p<0.05$). Meanwhile, decreased GSH levels improved in the group treated with IN ($p<0.05$, Figure 1a). MDA level measured as the degradation product of lipid peroxidation in intestinal tissue was significantly higher in MTX group than the control group ($p<0.01$), and these levels reduced after IN treatment ($p<0.05$; Figure 1b).

In addition, MPO activity, which is accepted as an indicator of neutrophil infiltration, was significantly higher in MTX group than the control group ($p<0.001$). IN treatment remarkably decreased the activity of the MPO released from the neutrophils ($p<0.001$; Figure 2a). $\mathrm{Na}^+\mathrm{K}^+\mathrm{ATPase}$ activity in the rat tissue was significantly lower in the MTX group than the control group ($p<0.01$). It was observed that IN treatment significantly prevented this decrease ($p<0.05$, Figure 2b).

A significant increase in both luminol and lucigenin levels was observed as an indicator of the reactive oxygen species in tissue damage ($p<0.001$). Contrarily, addition of IN to the treatment of rats under MTX treatment was found to cause a remarkable decrease in these reactive oxygen species ($p<0.01$; Figures 3a, b).

While epithelial structure, goblet cells, and gland structures were smooth in control and IN groups (Figures 4a, b), advanced loss of epithelium, increase in leukocytes, and hypertrophy in goblet cells were observed in MTX group (Figure 4c). A significant increase in epithelium regeneration, improvement in goblet cell morphology, and increase in mitosis were observed in MTX + IN group (Figure 4d).

Table 1. Serum TNF-$\alpha$ and IL-1$\beta$ values of control, IN, MTX and MTX-IN groups in MTX-induced intestinal mucositis model in rats

<table>
<thead>
<tr>
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<th>Control</th>
<th>IN</th>
<th>MTX</th>
<th>MTX-IN</th>
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<tr>
<td>TNF-$\alpha$ (pg/mL)</td>
<td>49.52 ± 2.71</td>
<td>42.82 ± 2.63</td>
<td>71.95 ± 3.92***</td>
<td>55.40 ± 3.11++</td>
</tr>
<tr>
<td>IL-1$\beta$ (pg/mL)</td>
<td>357 ± 22</td>
<td>316 ± 17</td>
<td>471 ± 28 ***</td>
<td>356 ± 11++</td>
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Comparisons *** $p<0.001$, according to the control group; ++ $p<0.01$, according to the MTX group.
Figure 2 a-b. a) MPO and b) Na+-K+ATPase activities of the intestinal tissue in control, IN, MTX and MTX-IN groups in MTX-induced intestinal mucositis model in rats. Comparisons *** p<0.01, according to the control group; + p<0.05, +++ p<0.001, according to the MTX group.

Figure 3 a-b. a) Luminol and b) lusigenin values of the intestinal tissue in control, IN, MTX and MTX-IN groups in MTX-induced intestinal mucositis model in rats. Comparisons ** p<0.01, *** Comparisons with p<0.001 according to the control group, ++ p<0.01 according to the MTX group.

Figure 4 a. Control group, smooth epithelium (arrow) goblet cells (arrowheads) and gland structures (*)

Figure 4 b. IN group, regularly formed goblet cells (arrowheads), epitelyum (arrow) and gland morphology (*)
Discussion

Intestinal mucositis is one of the side effects of antimitotic drugs used in the treatment of cancer patients. This toxic effect can cause dose limitations or stopping the cancer treatment (17). There are no current methods to prevent the development of intestinal mucositis because its pathophysiology is not yet clearly revealed.

Methotrexate treatment is among the frequently acknowledged causes of oral and intestinal mucositis in patients under cancer chemotherapy. Methotrexate exerts its toxicity by inhibiting dihydrofolate reductase enzyme, decreasing intracellular folate levels, and disrupting DNA synthesis (18). The cytotoxic effect of MTX is not selective to cancer cells. Toxicity is more frequently observed in normal tissues which proliferate fast, such as hematopoietic system, bone marrow, and gastrointestinal system mucosa (19). Among the MTX-related toxic effects, mucositis and enterocolitis causes malabsorption, diarrhea and pain (20).

Clinical studies performed in the last 30 years clearly show that dietary styles comprising formulas including high doses of specific immunonutrients improve the host response against damage due to its immunomodulatory, anti-inflammatory, anabolic and tissue preservative effects when compared to standard nutrition formulas or traditional treatment methods. Some researchers also emphasized how these nutrients improve host defense mechanisms and modulate inflammatory response in their studies (9-11, 21, 22).

One of the immunonutrients, arginine, was shown to increase wound healing while removing free radicals from the body, and have positive effects on decreased villus in the intestine, damaged crypt, inflammatory changes, cell death and MPO activity (23). Omega-3 fatty acids were shown to prevent tissue damage by exerting their anti-inflammatory and antithrombic effects, and help mucosal recovery (24). Dietary nucleotides, which are among the immunonutrients, are known to improve immune function upon being used by fast-proliferating cells such as T cells, macrophages and enterocytes, and increase resistance against infections. In a study performed in newborns receiving nucleotide supplement, it was observed that intestinal mucosal integrity was preserved and microflora was regulated (25). In this study, oral formula, which includes a combination of each of the three immunonutrients, was used, and decreasing the effects of MTX-dependent intestinal mucosal damage to a minimum was aimed.

Furthermore, TNF-α and IL-1β are among the important cytokines released from macrophages. Besides their cytotoxic effects, they have an important role in the regulation of inflammatory reaction and inflammation (26). In the study by Logan et al. (27), inflammatory response was higher in rats under
MTX treatment. This was explained by decreased barrier functions of pro-inflammatory cytokines such as TNF-α and IL-1β during the amplification phase of intestinal mucositis, disruption of epithelial integrity, and changes in mucus release. In this study, plasma levels of pro-inflammatory cytokines after MTX treatment was significantly high, and this increase was significantly prevented by IN treatment. This result suggested that the activation and infiltration of neutrophils which have a triggering role in tissue response can be prevented by oral IN formula and thus, inflammatory response can be decreased.

Glutathione is an antioxidant used in the measurement of oxidative stress. GSH reacts with free radicals and peroxides, converts them to harmless products and thereby prevents the cells from oxidative damage (28). Mucositis and malnutrition which occur after MTX treatment are correlated with increased intestinal permeability, inflammatory response, intestinal proteolysis in rat intestine and decreased GSH concentration (29, 30). In this study, oral IN formula was found to prevent rat intestine against the oxidative damage and increase GSH concentration in rat jejunal tissue.

Malondialdehyde is one of the frequently used indicators of oxidative damage (31). MDA levels were shown to increase as an indicator of lipid peroxidation in MTX-dependent intestinal damage (32). In this study, oral IN formula was shown to prevent the increase in MDA levels.

Myeloperoxidase levels in the intestine are indicators of neutrophil infiltration and acute inflammation (33). In addition, MPO enzyme released from neutrophils increases tissue damage and causes the formation of more free radicals (34, 35). In this study, it was observed that oral IN formula prevents increases in MPO levels and activation of neutrophils, which together trigger the oxidative mechanism in MTX-dependent intestinal damage.

Na⁺- K⁺ ATPase is an important phospholipid-dependent membrane enzyme which has a key role in cell structure and physiology due to its ability to maintain sodium and potassium gradient in the basolateral membrane of enterocytes, which is important for the absorption of nutrients (36). The increase in free oxygen radicals causes lipid peroxidation, which subsequently leads to the malfunction of erythrocyte membrane system and therefore Na⁺- K⁺ ATPase inactivation (37, 38). Based on the results of this study, oral IN formula helps the preservation of normal enterocyte structure and physiology.

Reactive oxygen radicals are formed in many inflammatory diseases and tissue damage (39, 40). Luminol is a technique used for H₂O₂, OH⁻, hypochlorite, peroxynitrite, and lipid peroxyl radicals, whereas lucigenin is selective for superoxide radicals (16). In this study, FOR levels in the jejunum tissue were measured using chemoluminescence method, and luminol and lucigenin levels decreased upon administration of oral IN formula. Thus, it was found that oral IN formula had antioxidant properties in the jejunal tissue samples.

Mucositis is histologically characterized by villus atrophy, enterocyte damage, and infiltration of inflammatory cells. Although these histological changes indicate epithelium loss-of-function, digestion and absorption capacity of enterocytes in mucositis is not known (41-43). In this study, in rats under MTX treatment, presence of irregular, even vacuolated enterocytes were observed in addition to villus atrophy, which is one of the typical histological symptoms of mucositis. Goblet cells were hypertrophic and accumulated at the top of the villi. A significant amount of epithelium was shed and inflammatory cells invaded the stroma of the villi. These characteristics of mucositis were also identified by other researchers (42-46). In this study, upon administering an oral IN formula to the rats, we observed that epithelium and goblet cells regenerated, villus structure recovered, and mitosis increased in enterocytes, and that oral IN formula increased cell proliferation in MTX-induced mucosal damage, thereby, reversing intestinal damage and inducing intestinal regeneration.

In conclusion, oral IN formula reverses MTX-associated intestinal damage in rats and induces intestinal recovery, and several factors are involved in the development of MTX-dependent mucositis. Therefore, there is a continuing pursuit of alternative treatment principles. Based on our study results, which is the first on this topic, we suggest that the application of oral IN formulation, a previously unused approach in the prevention of MTX-dependent intestinal mucositis, can enhance the success of the treatment and establish a new treatment principle in the clinical practice.
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Correspondence:
Elvan Yilmaz Akyuz
University of Health Sciences, Faculty of Health Sciences,
Department of Nutrition and Dietetics,
Istanbul/Turkey
E-mail: yilmaz.elvan@gmail.com