Progress in Nutrition 2018; Vol. 20, N. 2: 248-256 DOI: 10.23751/pn.v20i2.6396

Original article

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

Elvan Yilmaz Akyuz[1], Cebrail Akyuz[2], Gul Kiziltan[3], Ahmet Ozer Sehirli[4], Sule Cetinel[5]

[1] University of Health Sciences, Faculty of Health Sciences, Department of Nutrition and Dietetics, Istanbul/Turkey - E-mail: yilmaz.elvan@gmail.com; [2]Haydarpasa Numune Education and Research Hospital, Department of Surgery, Istanbul/Turkey; [3]Baskent 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 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 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 K_2HPO_4 (pH=7.4) and homogenized, and then centrifuged at 12,000 rpm for 10 min at 4°C. Samples were homogenized using 50 Mm K_2HPO_4 which includes an equivalent volume of 0.5% hexadecyltrimethylammonium hydroxide, and the MPO activity was measured by spectrophotometric measurement of the H_2O_2-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 Na_2HPO_4 $2H_2O$ 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 proinflammatory cytokines (TNF-α and IL-1β) 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). Na^+, K^+-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).

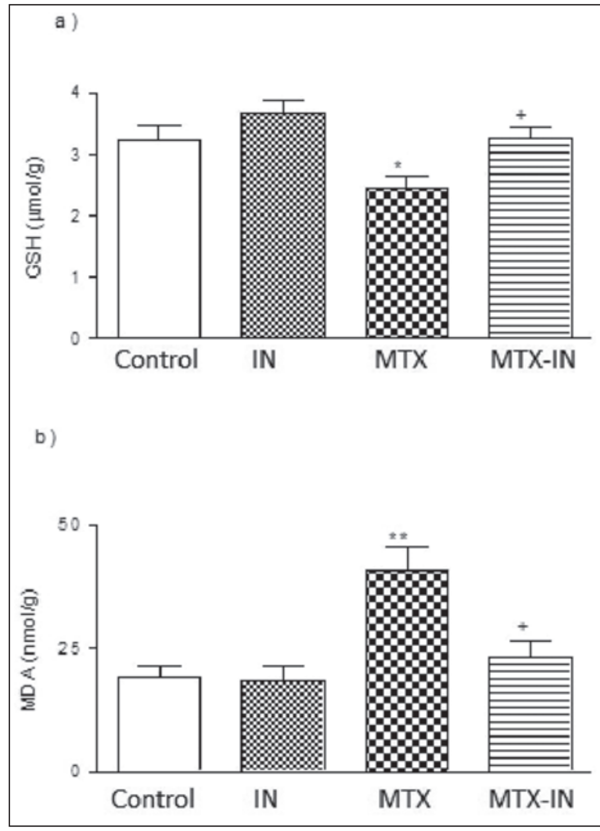

Figure 1 a-b. a) GSH and b) MDA values of the intestinal tissue in control, IN, MTX and MTX-IN groups in MTX-induced intestinal mucositis model in rats
Comparisons *** p<0.05, according to the control group;
++ p<0.01, according to the MTX group.

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-α and IL-1β values of control, IN, MTX and MTX-IN groups in MTX-induced intestinal mucositis model in rats

	Control	IN	MTX	MTX-IN
TNF-α (pg/mL)	49.52 ± 2.71	42.82 ± 2.63	71.95 ± 3.92***	55.40 ± 3.11++
IL-1β (pg/mL)	357 ± 22	316 ± 17	471 ± 28 ***	356 ± 11++

Comparisons *** p<0.001, according to the control group; ++ p<0.001, according to the MTX group

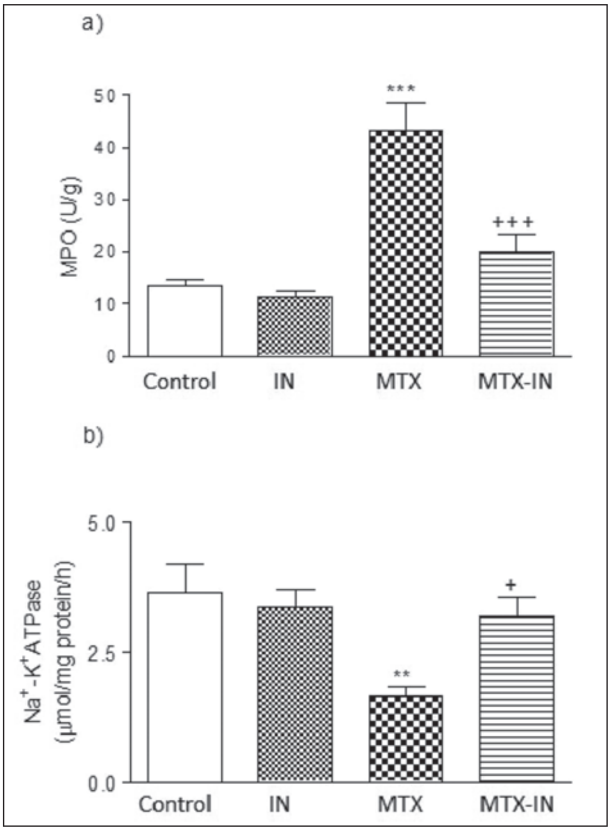

Figure 2 a-b. a) MPO and b) Na+-K+ATPase activities of the intestinal tissue in control, IN, MTX and MTX-IN groups in MTX-induced intestinal mucositis model in rats.
Comparisons *** p<0.01, according to the control group;
+ p<0.05, +++ p<0.001, according to the MTX group.

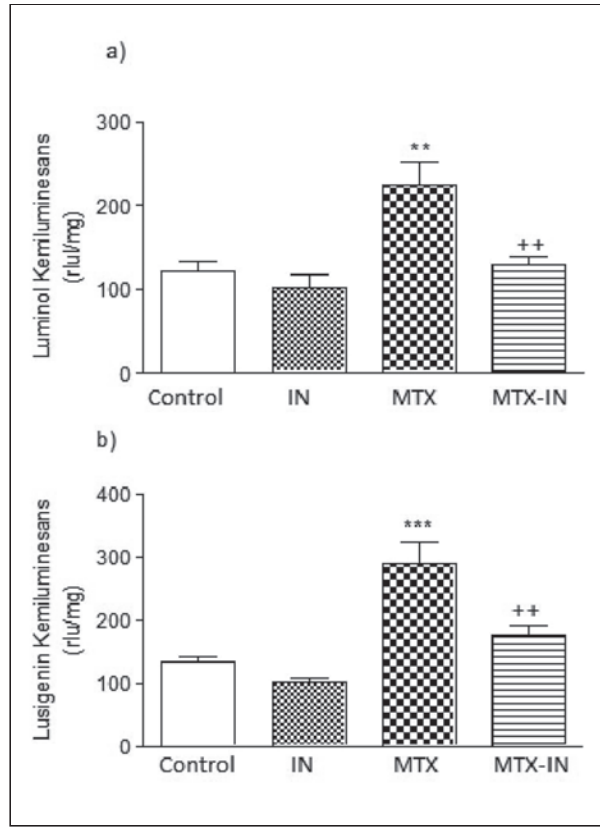

Figure 3 a-b. a) Luminol and b) lusigenin values of the intestinal tissue in control, IN, MTX and MTX-IN groups in MTX-induced intestinal mucositis model in rats
Comparisons ** p<0.01, *** Comparisons with p<0.001 according to the control group, ++ p<0.01 according to the MTX group.

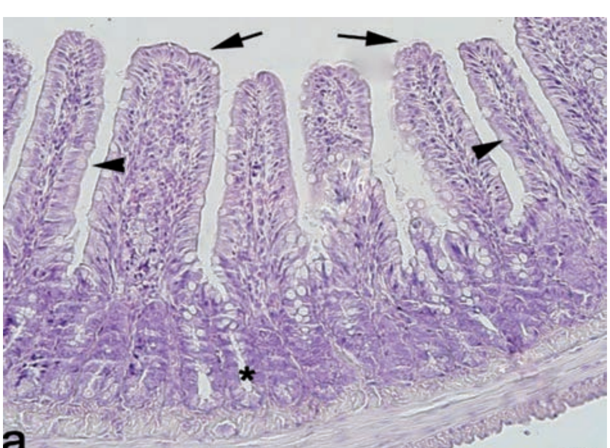

Figure 4 a. Control group, smooth epithelium (arrow) goblet cells (arrowheads) and gland structures (*)

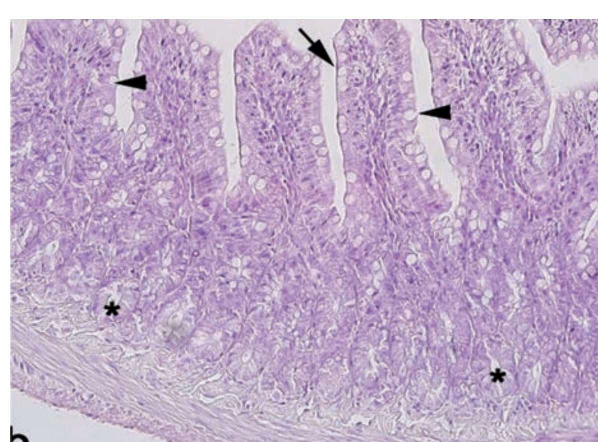

Figure 4 b. IN group, regularly formed goblet cells (arrowheads), epitelyum (arrow) and gland morphology (*)

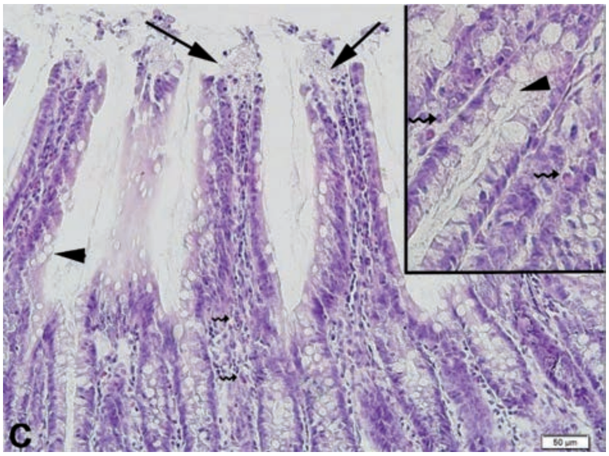

Figure 4 c. MTX group, severely shed epithelium (arrow) and hypertrophic goblet cells and leukocytes (dashed arrows)

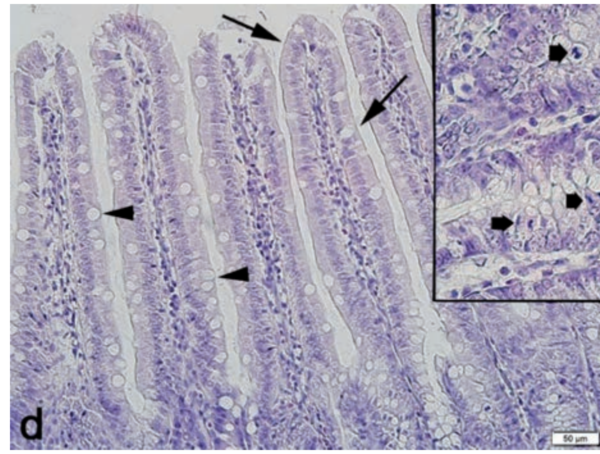

Figure 4 d. MTX+IN group, regenerated epithelium structure (arrow) and goblet cells (arrowheads), and cells undergoing active mitosis (bold arrows)

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 mucous 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 H_2O_2, 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.

Acknowledgement

This research was presented as a verbal statement at the 9th KEPAN congress. The authors have declared no conflicts of interest. All authors have read and approved the final manuscript, and agree to be accountable for all aspects of work ensuring integrity and accuracy. This research did not receive any specific grant from funding agencies in the public, commercial, or non-profit sectors.

References

1. Duncan M, Grant G. Review article: oral and intestinal mucositis causes and possible treatments. Aliment Pharmacol Ther 2003; 18: 853-874.
2. Niscola P, Romani C, Cupelli L, Scaramucci L, Tendas A, Dentamaro T, et al. Mucositis in patients with hematologic malignancies: an overview. Haematologica 2007; 92: 222-231.
3. Fijlstra M, Rings EH, Verkade HJ, Van Dijk TH, Kamps WA, Tissing WJ. Lactose maldigestion during methotrexate-induced gastrointestinal mucositis in a rat model. Am J Physiol Gastrointest Liver Physiol 2011; 300: 283-291.
4. Keefe DM, Schubert MM, Elting LS, Sonis ST, Epstein JB, Raber-Durlacher JE, et al. Updated clinical practice guidelines for the prevention and treatment of mucositis. Cancer 2007; 109: 820-31.
5. Sonis ST, Elting LS, Keefe D, Peterson DE, Schubert M, Hauer-Jensen M, et al. Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. Cancer 2004; 100: 1995-2025.
6. Giger U, Büchler M, Farhadi J, Berger D, Hüsler J, Schneider H, et al. Preoperative immunonutrition suppresses perioperative inflammatory response in patients with major abdominal surgery-a randomized controlled pilot study. Ann Surg Oncol. 2007; 14: 2798-806.
7. Alexander JW. Immunonutrition: the role of omega-3 fatty acids. Nutrition 1998; 14: 627-33.
8. Weimann A, Bastian L, Bischoff WE, Grotz M, Hansel M, Lotz J, et al. Influence of arginine, omega-3 fatty acids and nucleotide-supplemented enteral support on systemic inflammatory response syndrome and multiple organ failure in patients after severe trauma. Nutrition 1998; 14: 165-72.
9. Kemen M, Senkal M, Homann HH, Mumme A, Dauphin AK, Baier J, et al. Early postoperative enteral nutrition with arginine-omega-3 fatty acids and ribonucleic acid-supplemented diet versus placebo in cancer patients: an immunologic evaluation of impact. Crit Care Med 1995; 23: 652-9.
10. Daly JM, Lieberman MD, Goldfine J, Shou J, Weintraub F, Rosato EF, et al. Enteral nutrition with supplemental arginine, RNA, and omega-3 fatty acids in patients after operation: immunologic, metabolic, and clinical outcome. Surgery 1992; 112: 56-67.
11. Gianotti L, Braga M, Nespoli L, Radaelli G, Beneduce A, Carlo VD. A randomized controlled trial of preoperative oral supplementation with a specialized diet in patients with gastrointestinal cancer. Gastroenterology 2002; 122: 1763-70.
12. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animals tissues by thiobarbituric acid reaction. Anal Biochem 1979; 95: 351-358.
13. Hillegass LM, Griswold DE, Brickson B, Albrightson-Winslow C. Assessment of myeloperoxidase activity in whole rat kidney. J Pharmacol Methods 1990; 24: 285-95.
14. Beutler E. Glutathione in red blood cell metabolism. A manual of biochemical methods. New York: Grune&Stratton, 1975. p. 112-4.
15. Reading HW, Isbir T. The role of cation-activated ATPases in transmitter release from the rat iris. Q J Exp Physiol Cogn Med Sci 1980; 65:105-116.
16. Haklar G, Yüksel M, Yalçın AS. Chemiluminescence in the measurement of free radicals: theory and application on a tissue injury model. Marmara Medical Journal 1998; 11: 56-60.
17. Leblond J, Le Pessot F, Hubert-Buron A Duclos C, Vuichoud J, Faure M, et al. Chemotherapy-induced mucositis is associated with changes in proteolytic pathways. Exp Biol Med (Maywood) 2008; 233: 219-228.
18. Carneiro-Filho BA, Lima IP, Araujo DH, Cavalcante MC, Carvalho GH, Brito GA, et al. Intestinal barrier function and secretion in methotrexate-induced rat intestinal mucositis. Dig Dis Sci. 2004; 49: 65-72.
19. Nagakubo J, Tomimatsu T, Kitajima M, Takayama H, Aimi N, Horie T. Characteristics of transport of fluoresceinated methotrexate in rat small intestine. Life Sci 2001; 69: 739-47.
20. Van't Land B, Blijlevens NM, Marteijn J, Timal S, Donnelly JP, de Witte TJ, et al. Role of curcumin and the inhibition of nf-kappaβ in the onset of chemotherapy-induced mucosal barrier injury. Leukemia 2004; 18: 276-84.
21. Braga M, Vignali A, Gianotti L, Cestari A, Profili M, Carlo VD. Immune and nutritional effects of early enteral nutrition after major abdominal operations. Eur J Surg 1996; 162: 105-12.
22. Senkal M, Kemen M, Homann HH, Eickhoff U, Baier J, Zumtobel V. Modulation of postoperative immune response by enteral nutrition with a diet enriched with arginine, RNA, and omega-3 fatty acids in patients with upper gastrointestinal cancer. Eur J Surg 1995; 161: 115-22.
23. Leitão RF, Brito GA, Oriá RB, Braga-Neto MB, Bellaguarda EA, Silva JV. Role of inducible nitric oxide synthase pathway on methotrexate-induced intestinal mucositis in rodents. BMC Gastroenterol 2011; 11:90.
24. Koppelmann T, Pollak Y, Mogilner J, Bejar J, Coran AG, Sukhotnik I. Reversal of severe methotrexate-induced intestinal damage using enteral n-3 fatty acids. Br J Nutr 2013; 109: 89-98.
25. Yu VY. Scientific rationale and benefits of nucleotide supplementation of infant formula. J Paediatr Child Health 2002; 38: 543-9.

26. Hillegass LM, Griswold DE, Brickson B, Albrightson-Winslow C. Assessment of myeloperoxidase activity in whole rat kidney. J Pharmacol Methods 1990; 24: 285-95.
27. Logan RM, Gibson RJ, Bowen JM, Stringer AM, Sonis ST, Keefe DM. Characterisation of mucosal changes in the alimentary tract following administration of irinotecan: implications for the pathobiology of mucositis. Cancer Chemother Pharmacol 2008; 62: 33-41.
28. Urso ML, Clarkson PM. Oxidative stress, exercise and antioxidant supplementation. Toxicol 2003; 189: 41-54.
29. Lyoumi S, Tamion F, Petit J, Déchelotte P, Dauguet C, Scotté M. Induction and modulation of acute-phase response by protein malnutrition in rats: comparative effect of systemic and localized inflammation on interleukin-6 and acute-phase protein synthesis. J Nutr 1998; 128: 166-174.
30. Belmonte L, Coeffier M, Le Pessot F, Miralles-Barrachina O, Hiron M, Leplingard A, et al. Effects of glutamine supplementation on gut barrier, glutathione content and acute phase response in malnourished rats during inflammatory shock. World J Gastroenterol 2007; 13: 2833-2840.
31. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007; 39: 44-84.
32. Ciralik H, Bulbuloglu E, Cetinkaya A, Kurutas EB, Celik M, Polat A. Effects of N-acetylcysteine on methotrexate-induced small intestinal damage in rats. Mt Sinai J Med 2006; 73: 1086-92.
33. Howarth GS, Francis GL, Cool JC, Xu X, Byard RW, Read LC. Milk growth factors enriched from cheese whey ameliorate intestinal damage by methotrexate when administered orally to rats. J Nutr 1996; 126: 2519–2530.
34. Yılmaz HR, Uz E, Yucel N, Altuntas İ, Ozcelik N. Protective effect of caffeic acid phenethyl ester (CAPE) on lipid peroxidation and antioxidant enzymes in diabetic rat liver. J Biochem Molecular Toxicology 2004; 18: 234-238.
35. Akyüz C, ehirli AO, Topaloğlu U, Velioglu Ogunc A, Cetinel S, Sener G. Protective effects of proanthocyanidin on cerulein-induced acute pancreatic inflammation in rats. Gastroenterology Research 2009; 2: 20-28.
36. Persson J. Alcohol and the small intestine. Scand J Gastroenterol 1991; 26: 3-15.
37. Cuzzocrea S, Reiter RJ. Pharmacological action of melatonin in shock, inflammation and ischgemia/ reperfusion injury. Eur J Pharmacol 2001; 426: 1-10.
38. Ilhan N, Halifeoglu I, Ozercan HI, Ilhan N. Tissue malondialdehyde and adenosine triphosphatase level after experimental liver ischaemia–reperfusion damage. Cell Biochem Funct 2001; 19: 207-12.
39. Grisham MB, Volkmer C, Tso P, Yamada T. Metabolism of trinitrobenzene sulfonic acid by the rat colon produces reactive oxygen species. Gastroenterology 1991; 101: 540-547.
40. Siems WG, Grune T, Werner A, Gerber G, Buntrock P, Schneider W. Protective influence of oxypurinol on the trinitrobenzene sulfonic acid (TNB) model of inflammatory bowel disease in rats. Cell Mol Biol 1992; 38: 189-199.
41. Sonis ST, Elting LS, Keefe D, Peterson DE, Schubert M, Hauer-Jensen M, et al. Perspectives on cancer therapy-induced mucosal injury: pathogenesis, measurement, epidemiology, and consequences for patients. Cancer 2004; 100: 1995-2025.
42. Taminiau JA, Gall DG, Hamilton JR. Response of the rat small intestine epithelium to methotrexate. Gut 1980; 21: 486-492.
43. Tooley KL, Howarth GS, Lymn KA, Lawrence A, Butler RN. Oral ingestion of streptococcus thermophilus diminishes severity of small intestinal mucositis in methotrexate treated rats. Cancer Biol Ther 2006; 5: 593-600.
44. De Koning BAE, Lindenbergh-Kortleve DJ, Pieters R, Rings EHHM, Buller HA, Renes I.B, et al. The effect of cytostatic drug treatment on intestine-specific transcription factors Cdx2, GATA-4 and HNF-1alpha in mice. Cancer Chemother Pharmacol 2006;57: 801-810.
45. Verburg M, Renes IB, Van Nispen DJ, Ferdinandusse S, Jorritsma M, Büller HA, et al. Specific responses in rat small intestinal epithelial mRNA expression and protein levels during chemotherapeutic damage and regeneration. J Histochem Cytochem 2002; 50: 1525-1536.
46. Lindsay RJ, Geier MS, Yazbeck R, Butler RN, Howarth GS. Orally administered emu oil decreases acute inflammation and alters selected small intestinal parameters in a rat model of mucositis. Br J Nutr 2010; 104: 113-119.

Correspondence:
Elvan Ylmaz Akyuz
University of Health Sciences, Faculty of Health Sciences,
Department of Nutrition and Dietetics,
Istanbul/Turkey
E-mail: yilmaz.elvan@gmail.com