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ARAŞTIRMA / RESEARCH

Effects of glutamine and β-hydroxy β-methyl butyrate on methotrexate induced intestinal mucositis

Glutamin ve β-hidroksi β-metil bütiratin metotreksatin indüklediği intestinal mukozit üzerine etkisi

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Abstract

Purpose: We aimed to evaluate preventing effects of Glutamine (Gln) and β-hydroxy β-methylbutyrate (HMB) on methotrexate induced intestinal mucositis
Materials and Methods: Fifty Wistar albino rats were divided into 5 groups (G). Group G1 defined as control and G2 was the methotrexate (MTX) treated group. The group G3, G4 and G5 were MTX plus Gln, Mtx plus HMB, MTX plus Gln and HMB respectively. Distilled water was applied by gavage to the first 2 groups. Rat received intraperitoneal injections of MTX at the third day. On the fifth day intestinal tissue samples were obtained.
Results: The G5 had similar results with the G1 group in the crypt length. According to Park scoring, G1 and G5 were both the highest scores among five study groups. When the tissue was inspected by caspase-3 coating, the lowest apoptotic rate is found in the G5.
Conclusion: This research showed that the combination of Gln and HMB use is more effective then the separate use of both chemicals.
Keywords:. Glutamine, β-hydroxy β-methylbutyrate, mucositis, methotrexate, rat

Öz

Amaç: Metotreksatın sebep olduğu intestinal mukozit üzerinde Glutamin (Gln) ve β-hidroksi β-metilbutiratın (HMB) önleyici etkilerini değerlendirmeyi amaçladık
Gereç ve Yöntem: 50 Wistar albino cinsi sıçan 5 gruba (G) ayrıldı. G1 kontrol grubu ve G2 metotreksat (MTX) ile tedavi edilen gruptu. G3, G4 ve G5 sırasıyla MTX ve Gln, Mtx ve HMB, MTX ve Gln, HMB verilen gruplardı. Sıçanlara üç gün intraperitoneal MTX uygulandı. Beşinci günde bağırsak doku örnekleri alındı.
Bulgular: G5 kript derinlik ölçümleri G1 grubu ile benzerdi. Park skorlamasına göre G1 ve G5, beş çalışma grubu arasında en yüksek skoru alan gruplardı. Doku kaspaz-3 ile incelendiğinde, en düşük apoptotik oran G5'te bulundu.
Sonuç: Bu çalışma, Gln ve HMB kullanım kombinasyonunun her iki kimyasal maddenin ayrı kullanımından daha etkili olduğunu göstermiştir

Anahtar kelimeler: Glutamin, β-hidroksi β-metilbutirat, mukozit, methotreksat, rat

INTRODUCTION

Mucositis is one of the most common and severe side effects seen after cytotoxic chemotherapy and radiotherapy. It is a separate condition that should be treated in acute toxicity due to treatment. It can range from mild sensitivity to multiple combined ulcers and bleeding lesions. All cytotoxic drugs cause apoptosis in crypt cells, followed by crypt hypoplasia and subsequently reactive crypt hyperplasia.

Methotrexate (MTX) is the most widely used antimetabolite in oncology[1]. The mucosa of the digestive tract, particularly that of the small intestine, is a critical site of action of anticancer agents

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including MTX, as the undifferentiated epithelial cells in the crypts, which ensure continuous and relatively fast regeneration of the epithelium, are particularly sensitive to these cytostatic drugs[1-3].

Nutritional interventions may be beneficial to limit chemotherapy-induced intestinal mucositis. Glutamine (Gln) is a conditionally essential amino acid during various conditions of stress[4]. Gln is depleted in cancer patients and the content of Gln is reduced during intestinal inflammation[5-6]. Gln prevents atrophy of the intestinal mucosa in patients receiving total parenteral nutrition and modulates intestinal inflammatory and antioxidant responses[7-8]. Beta-hydroxy-beta-methylbutyrate (HMB), a leucine metabolite, has been shown to be anti-catabolic and effective at attenuating muscle atrophy during exercise stress, and in models of cancer[9-11]. HMB also have an action important to wound closure and healing[12]. However, its effects on chemotherapy-induced gastrointestinal mucositis have not been examined yet.

The aim of this study was to investigate and compare the effect of Gln and HMB in preventing intestinal mucositis and in stimulating enterocyte turnover after MTX induced intestinal damage in a rat model. In as much as Gln and HMB enhances proliferation and reduces apoptosis, we tested whether it would accelerate cell turnover and improve intestinal recovery in this model.

MATERIALS AND METHODS

Animals

The study included 25 adult female and 25 adult male Wistar albino rats (250-300 g). Animals were housed four per cage in a controlled animal holding room with a 12/12-h light/dark cycle; temperature and relative humidity were continually monitored to provide standard laboratory conditions. Food and water were provided ad libitum. In this study, animal rights were respected in accordance with the principles of Animals Guide for the Care and Use of Laboratory Animals. The study protocol was approved by the Animal Care and Ethics Committee of the Mersin University number of 19/04/2012 2012-15.

Experimental design

The rats were randomly divided into 5 groups according to the sex (G). Control rats (G1) treated with normal saline (1ml) given intraperitoneally (IP) one dose (n=10), MTX rats (G2-5) were treated single dose (20 mg/kg) of MTX given IP. All IP injections were done at 72 hours of experiment. G1 and MTX group (G2) treated with 6 ml/kg normal saline via gavage for 5 days. MTX and Gln group (G3) treated with 1 g/kg L-Glutamine (Resource Glutamine®, Nestle, Nutrition, Swisszerland) via gavage for 5 days. MTX and HMB group (G4) treated with HMB (Dymatize Enterprises, Dallas, TX, USA) 200 mg/kg via gavage for 5 days. MTX-Gln-HMB group (G5) was treated with both Gln and HMB via gavage for 5 days with the same doses. Three days after either MTX or saline injection, bromodeoxyuridine (BrdU) at 50 mg/100g body weight injected IP and 2 hour later rats were anesthetized with IP 90 mg/kg ketamine hydrochloride and 10 mg/kg xylazine. Liquid and food consumption, weight gain and loss, diarrhea were monitored daily in rats for 5 days. The intestine tissues were used from sacrificed rat at day 5.

Tissue collection and histological evaluation

To determine the intestinal damage, crypt depth and PARC scoring were measured by histochemical methods. Jejuna tissues were fixed with 10% formalin, routinely processed for light microscope and embedded in paraffin blocks. Sections (4µm) were cut by microtome and stained with hematoxyline and eosin. Slides were examined by an Olympus BX50 light microscope and photographed by a Nikon Cool-Pix 5000 digital camera attached to the microscope.

In order to immunohistochemical labeling for BrdU and active caspase-3, sections were deparaffinized and rehydrated. Following antigen retrieval, sections were incubated with 2N HCl for 30 minutes at 37 ^{0}C for DNA denaturation. Acid neutralization was performed by 0.1 M di-sodium tetra borate. For BrdU labeling, 1/20 diluted anti BrdU mouse monoclonal antibody (Cat. No: NCL-BrdU Novocastra, Leica Biosystems, Wetzlar, Germany) dropped on the sections and incubated overnight at +4 ^{0}C. For active caspase-3 immunohistochemistry, acid denaturation and neutralisation steps were omitted and 1/400 diluted rabbit polyclonal anti active caspase-3 antibody (Kat. no: 9661S, Cell Signaling Technology Inc. Danvers, MA, ABD) was dropped on the sections and incubated overnight at +4 ^{0}C. The following day, sections were incubated with biotinylated secondary goat anti rabbit antibody

(Labvision Corp., TP-125-BN, Fremont, CA) at room temperature for 1 hour. 3,3'-diaminobenzidine tetra-hydrochloride (DAB) was used for visualization of antibody bounding. The sections were counterstained with hematoxylin. Buffer including 0.5% bovine serum albumin (BSA) and no primary antibody was dropped on the sections separated for negative control. Images were captured using the same equipment above.

For evaluating crypt depths of the groups, in hematoxyline and eosin stained paraffin sections, 10 villi were randomly selected and depth of 10 intestinal crypts on these villi were measured by a commercially available software (ITEM 5.0© Soft Imaging Systems®).

The Intestinal mucosal damage was analyzed on a grading scale from 0 to 8 as described previously by Park et.al[13].

0: Normal mucosa

1: Subepithelial space formation at the villus tip

2: Increased subepithelial area

3: epithelial detachment along the entire villus margin

4: Peeling in villus (loss of epithelium)

5: Loss of villus tissue

6: Infarct in crypt plate

7: Infarction throughout the entire mucosa

8: It was scored in the form of infarct, along the entire intestinal wall.

Statistical analysis

The MedCalc statistical programme was used for the analyses. Shapiro Wilk test was used for normal distribution of BrdU (+) and (-) cell count and PARC scoring. Kruskall Wallis test was used for PARK scoring to comparing the groups. Oneway ANOVA and post hock Tukey were used in accordance to comparing the differences between all groups. Repeated ANOVA test was used for comparing the differences of body mass between groups.

RESULTS

No findings appeared to require any termination of the experiment clinically. Diarrhea appeared on the 3rd day of the experiment in all groups except the G1 (control group). The fifth female rat died in G4 group since an unknown effect.

In G1 histopathological surface and crypt epithelium were in regular morphology and villi had lamina propria. In G2, it was observed that reduced height and flattening of the villi and surface epithelium poured from the apical region into the crypt epithelium and inflammatory cell infiltration has increased very markedly. In G3 and G4, protection of some of the villi and a reduction in the number of inflammatory cell compared to G2 were seen. Villi characteristics in G5 showed similarity with G1, surface and crypt epithelium were renewed in many areas and inflammatory cell infiltration was observed to be significantly reduced in G5. The results of the comparison between-groups are shown in the Table 1.

The most significant reduction in crypt depth was determined in the G2 when crypt depth compared between the groups. There was no significant difference between G3 and G4 ($p > 0.05$); and also G1 and G5 results were similar (Table 1). The most damage was detected in G2 when mucosal damage evaluated by Park scoring. G1 showed no tissue damage. There was no significant difference between G3 and G4 ($p > 0.05$); but the G5 park scoring was more significant than G3 and G4 (Table 1).

When the cells labeled with BrdU evaluated, there was a significant reduction in G2 compared to G1 ($p<0.05$). A significant difference in counting cells labeled with BrdU was detected between G3 and G4 ($p<0.05$). The cells labeled with BrdU were highest in G5 ($p<0.05$). BrdU labeled cells was not determined difference between G3 and G1 ($p>0.05$) (Table 1).

As a result of caspase-3 immunohistochemical staining, the apoptotic and non-apoptotic cell counts calculated as a percentage; it was determined that G1 have lowest and highest apoptotic cell counts in G1 and G2 respectively ($p<0.05$). Apoptotic cell percentages gradually decrease in G3, G4 and G5. Among them, the lowest apoptotic cell percentage was detected in G5 ($p<0.05$) (Table1).

DISCUSSION

Proliferation and self-renewal of epithelial cells of the small intestine occurs in the crypts. After treatment with MTX, significantly damage occurs in the small intestine. In the study of Miyazono *et al*[14], the oxidative stress and especially neutrophil infiltration

has been shown to play a role in the damage that occurs after MTX treatment. Taminiau JA *et al*[15] showed that the most serious effects of histopathological changes caused by MTX were crypt and villous ablation in the proximal small intestine. Applying MTX causes an increase in apoptosis and peaked at 6 hours after the administration in rats. The most severe mucosal injury observed after about 72 hours in the studies of Gibson RJ *et al*[16], and Gulgun M *et al*[17]

Table 1. Methotrexate application and post-treatment microscopic mucosal parameters and serum total antioxidant capacity

Groups	Rat (n)	Crypt depth (mean± SD)	Park Score (mean± SD)	BrdU Positive Cell Number	Caspase-3 positive cell (%)
1	10	2.04 ±0.24	0.0 ± 0.0	89.00 ± 14.83	7.76 ± 2.85
2	10	1.11 ± 0.11	3.50 ± 0.85	52.62 ± 16.75	95.72 ± 16.04
3	10	1.40 ±0.15	2.30 ± 0.67	97.41 ± 21.02	66.98 ± 9.49
4	9	1.54 ±0.39	1.89 ± 0.60	139.56 ± 34.01	40.52 ± 9.42
5	10	1.84 ±0.11	1.0 ± 0.47	177.00 ± 33.13	22.06 ± 6.51
p1		0.000	0.000	0.018	0.0001
p2		0.000	0.000	0.943	0.0001
p3		0.000	0.000	0.001	0.0001
p4		0.033	0.036	0.0001	0.019
p5		0.001	0.001	0.002	0.0001
p6		0.000	0.000	0.0001	0.0001
p7		0.000	0.000	0.0001	0.0001
p8		0.308	0.304	0.006	0.0001
p9		0.000	0.000	0.0001	0.0001
p10		0.001	0.001	0.018	0.002

p1:the comparison between G1 and G2, *p2*: G1 and G3, *p3:* G1 and G4, *p4*: G1 and G5, , *p5*: G2 and G3, *p6*: G2 and G4, *:p7:* G2 and G5, *p8*: G3 and G4, *p9*: G3 and G5, *p10*: G4 and G5 ($p<0.05$ is statistically significant)

In this study, diarrhea was observed in all groups MTX applied. The diarrhea-preventive effect was not detected for Gln, HMB and Gln-HMB combinations. In the evaluation of crypt depth G2 was lowest, but the crypt depth of G5 was close to G1. This results showed us that the use of Gln and HMB in the MTX induced intestinal damage provides nearly normal gastrointestinal mucosa histological structure.

L-Gln indirectly participate protein synthesis in rapidly proliferating cells such as lymphocytes and enterocytes. It also served as a precursor molecule for the synthesis of DNA and glutathione. One important function is that it is an important source of energy in order to restructure and to maintain the healthy structure of the gastrointestinal tract[18]. In studies on both humans and animals, the effect of Gln gives conflicting results[19-20]. There are several studies showing that Gln support reduces development of mucositis depends on MTX and RT in rats[21-22]. The data obtained from this study support the literature and showed that Gln reduces the mucosal damage.

Similarly, use of HMB in an experimental study demonstrates that the positive effects on wound healing by increasing collagen synthesis and reduce the occurrence of damage[23]. HMB prevents protein degradation by reducing the effectiveness of the ubiquitin-proteasome system; also it assists the development of immune system function and wound healing[24] HMB prevents cell apoptosis by inhibiting the activation of caspase-8, and have also protective effect by increased synthesis of proteins and leading to the decline NF-ϰB mediated protein degradation[25-27]. HMB also increases the protein synthesis by

activating the mTOR pathway and show protective effect in the cell[28]. We believe that the improved detection of histological parameters in HMB group compared to the MTX group occurred through these mechanisms. The best results in our study were seen in the combination group treated with Gln and HMB. We also believe that both of Gln stimulated cellular proliferation and inhibitory HMB effects on the cell destruction provided our better results. In our study, using Gln and HMB reduce destruction of enterocytes and combined using make better results.

DNA synthesis and replication are indications of cellular proliferation. It is used in order to determine the cell proliferation utilizing a BrdU binding to DNA during replication. In our study, the proliferation index of enterocytes proliferation is determined using BrdU. The numbers of BrdU labeled cells were found the lowest in G2. The numbers of BrdU labeled cells were not statistically different between G1 and G3. However, the numbers of BrdU labeled cells were significantly increased in both groups treatment with HMB and Gln-HMB combination. We believed that the similarity between G1 and G3 is linked to stimulating proliferation effects of Gln. Comparing with G3, proliferation of enterocytes was found to be higher in G4. We believe that the HMB to reduce the effects of inflammatory cytokines therefore less devastation after MTX makes proliferation of enterocytes faster and easier. Using in combination Gln and HMB could have been better results because of additive effect.

Although some studies available about that Gln use in order to reduce the impact of the antineoplastic agents in chemotherapy to destruction of tissues and cells, there is no study showing HMB have protective effect on enterocytes[21,29].

In conclusion, no study has been observed about use of HMB with Gln in order to prevent development of MTX-induced intestinal mucositis. Because of the pioneering work done regarding this issue, our research have original features. Our results showed that combined therapy Gln and HMB gave better results than stand-alone use. Although various treatments applied until today, it has not been possible to prevent the formation of neither oral nor intestinal mucositis. Study data can conflict with each other; it shows that there is no definite treatment. We believe that these data we obtained should be confirmed in future clinical trials. In case of obtaining successful results, it might be possible to say that new horizons would be opened in the prevention of oral and intestinal mucositis and would make positive contributions to the treatment strategies. The limitations of the study were the fact that it was studied with a single center and a small number of rats, and that the genetic study was not shown at the cellular level.

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