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RESEARCH ARTICLE

The Effectiveness of Hesperidin in the Prevention of Bacterial Translocation Caused by Methotrexate in the Gastrointestinal Tract

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Summary

Methotrexate (MTX) is an antimetabolite that it is widely used in childhood cancers. Gastrointestinal toxicity stemming from oxidative damage is an important factor limiting its use. MTX causes morphological damage in the mucosa of the small intestine and serious barrier function disorder. Bacterial translocation can be seen when intestinal barrier functions are deteriorated. The aim of this study was to investigate the effect of hesperidin, a powerful antioxidant, in the prevention of bacterial translocation caused by MTX. Rats were given a single intraperitoneal dose of MTX at 20 mg/kg body weight. Hesperidin was given with oral gavage at 200 mg/kg body weight through 5 days. On the 6th day, biopsy specimens from the ileocecal region, ascending colon and mesenteric lymph nodes were placed in culture media. Increased intestinal bacteria growth was found and prominent bacterial translocation were determined in the MTX group ($P<0.05$). Hesperidin significantly reduced the growth load and bacterial translocation. This study showed that hesperidin protects against translocation by preventing damage caused by MTX.

Keywords: *Methotrexate, Bacterial translocation, Hesperidin*

Metotreksatın Neden Olduğu Gastrointestinal Kanaldan Bakteriyel Translokasyonun Önlenmesinde Hesperidinin Etkinliği

Özet

Metotreksatı (MTX) çocukluk çağı kanserlerinde yaygın olarak kullanılan antimetabolittir. Oksidatif hasardan kaynaklanan gastrointestinal toksisite MTX için önemli bir sınırlayıcı faktördür. MTX'in ince barsak mukozasında morfolojik hasar ve ciddi bariyer fonksiyon bozukluğuna neden olur. Barsak bariyer fonksiyonları bozulduğu zaman, bakteriyel translokasyon görülebilir. Bu çalışmanın amacı, MTX'in neden olduğu bakteriyel translokasyonun önlenmesinde güçlü bir antioksidan olan hesperidinin etkisini değerlendirmektir. Sıçanlara MTX tek doz intraperitoneal yoldan 20 mg/kg/gün verildi. İntestinal bakteriyel translokasyonu önlemek için ardışık 5 gün hesperidin 200 mg/kg/gün gavaj ile uygulandı. Çalışmanın 6. günü ileoçekal bölge, asendan kolon ve mesenterial lenf nodlarından biyopsi örnekleri kültür vasatlarına alındı. MTX grubunda, barsakta daha fazla üreme ve aynı zamanda belirgin bakteriyel translokasyon saptandı ($P<0.05$). Hesperidin üreme yükünü ve bakteriyel translokasyonu belirgin azalmaktadır. Bu çalışmada hesperidinin MTX'ın oluşturduğu hasarını önleyerek translokasyonu engellediği görülmüştür.

Anahtar sözcükler: *Metotreksat, Bakteriyel translokasyon, Hesperidin*

INTRODUCTION

MTX is the most widely used an anti-metabolite in cancer chemotherapy. It also plays a crucial role in the treatment of a range of diseases, including lymphocytic leukemia, non-Hodgkin's lymphoma, osteosarcoma, choriocarcinoma, head and neck cancer and breast cancer. MTX also has major toxic effects, including intestinal injury and enterocolitis. Administration of MTX compromises mucosal barrier function, leading to gut flora invading the circulation [1,2].

Mucosal injury and compromise of the intestinal barrier can result in nonspecific and unlimited translocation of intestinal microorganisms. Although MTX has been shown

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to cause morphological injuries included intestinal barrier function damage in the mucosa of small intestine its association with bacterial translocation is still unclear [3]. Reducing mucosal damage is important in order to lower the side-effects in patients receiving chemotherapy to a minimum. Neutrophil infiltration and oxidative stress have been shown to be involved in intestinal damage stimulated with MTX [4]. Recent studies have concentrated on antioxidant substances that may prevent the undesired side-effects of MTX in intestinal tissue. Several studies have been performed using antioxidant agents for the purpose of preventing MTX-related damage [5-8].

Hesperidin (flavanone) (HES) is a member of the bi-flavonoid group. It is a potent antioxidant with effects similar to those of Vitamin E and has various biological effects in the nervous systems of numerous mammals. In vitro and in vivo studies have demonstrated antimicrobial, antiviral, antihypertensive, hypolipidemic, antiulcerogenic, antineoplastic, anti-inflammatory, antioxidant and anti-hepatotoxic effects [9]. HES is reported to eliminate free oxygen radicals. It also inhibits the effects of pro-inflammatory mediators that induce neutrophil chemotaxis, such as prostaglandins [10]. In general, citrus bioflavonoids containing HES are known to be safe and have no side-effects, even in pregnancy [11].

This study was planned to determine the effect of HES on translocation that may occur as the result of an increase in certain bacterial groups and changes in the intestinal flora due to MTX on intestinal barrier functions.

MATERIAL and METHODS

Experimental Model

This study was planned in order to investigate the effect of HES on translocation that may occur with a rise in certain bacteria groups and the impairment of mucosal integrity caused by MTX. Forty age and race-matched 16-week-old male Wistar albino rats weighing 275-420 g were included. All rats were fed under identical conditions. Rats were housed 6 or 7 to a cage and maintained in a 12-hour light/dark cycle, at a constant temperature of 21±2°C and relative humidity of 40%-60%. They were divided into four groups: (1) a control group (n = 10), (2) rats receiving MTX alone (n = 10), (3) rats receiving HES alone (n = 10), and (4) rats receiving MTX plus HES (n = 10). All experimental protocols were approved by the Committee on Animal Research at Cukurova University, Turkey. Experimental animals were cared for and used in accordance with the National Institute of Health Guide (8.10.2012, Number - 1).

Study Protocol

Control Group: Serum saline was administered orally through an intragastric tube for five days.

MTX-Group: A single dose (20 mg/kg body weight) of MTX (MTX 500 mg in 20 ml vehicle, F.H. Faulding & Co. Ltd., Australia) was given intraperitoneally to each rat. Serum saline was applied as a placebo through intragastric tube, 5 days after MTX injection, and continued daily until the rats were sacrificed.

HES-Group: HES (SIGMA, USA) 200 mg/kg dissolved in distilled water was administered orally through an intragastric tube for five days and continued until rats were sacrificed.

MTX plus HES-Group: Five days after administration of a single intraperitoneal dose of MTX (20 mg/kg), HES (Hesperidin, 200 mg/kg body weight dissolved in distilled water) was administered orally through an intragastric tube every day and continued until the rats were sacrificed.

On the 6th day after injection of MTX, rats were sacrificed using intraperitoneal ketamine HCl (Ketalar, Parke Davis and Eczacıbaşı, Istanbul) (50 mg/kg) and xylazine HCl (Rompun, Bayer Health Care) (5 mg/kg body weight) injection anesthesia.

Microbiological Analysis

Specimens were taken to the laboratory within 1 h at +4°C in previously tared broth medium containing 1 ml Brain-Heart Infusion Broth (BHIB) and analyzed in terms of microbial load. Specimens brought to the laboratory were re-weighed, the weight of the biopsy specimens was recorded. Tissue samples were subsequently broken down in the BHIB with sterile glass rods. Specimens were weighed again at the end of the breaking down process. Specimens were homogenized by vortexing; 0.5 ml of specimen was taken and 1/10 dilutions in 4.5 ml BHIB were prepared. Subsequently, $10^{-2,-8}$ dilutions on a log10 base were obtained. In order to count bacteria in the form of cfu/ml from each dilution, 0.1 ml was inoculated into five McConkey broths, and the inoculated broths were left to incubate for 18 h at 37°C. Following incubation, mean colony numbers with a standard deviation ±10 from slides with countable colony numbers from 10 to 100 and determined as the figure for cfu/mg calculation. The figure was multiplied by the dilution coefficient, and ratios were determined first with the ml specimen and then with the tissue in ml, thus giving the figure in the mg tissue specimen. The study was based on Gram-negative bacteria colonization, regardless of species.

Statistical Analysis

Values were presented as means (minimum-maximum). Analysis of bacterial density data was performed by using SPSS-15. The Mann-Whitney U test was used to compare the results obtained from the four groups; $P<0.05$ was regarded as statistically significant.

RESULTS

Bacterial load was determined as cfu/g in the tissue samples. Although no bacterial growth was seen in any specimens, the highest value in the mesenteric lymph node specimens was 0.60 mg in the control group, The highest specimen weights and growing bacterial densities were 0.48 mg and $5.5X10^3$ cfu/g, in MTX group, 0.50 mg and 0 cfu/g in HES group, and 0.45 mg and $2x10^3$ cfu/ respectively g in MTX plus HES-Group *(Table 1)*.

The highest weight of ileocecal region was 0.31 mg in control group, and the highest bacterial density was 4.3 x 10^5 cfu/g in one specimen. Specimen weight and growing bacteria were found as 0.48 mg and 5.5 x 10^6 cfu/g in MTX group, 0.50 mg and $4.4x10^3$ cfu/g in HES group, 0.45 mg and 9.2 x 10^4 cfu/g respectively in MTX plus HES-Group *(Table 1)*.

The highest value of bacteriological load was found as 1.82 mg in the ascending colon, in control group, and the highest bacterial load was $5.5x10^5$ cfu/g. The highest weight and growing bacteria densities from this group specimens were 1.35 mg and 5.8 x 10^6cfu/g in MTX group, 1.18 mg and 2.7x 10^5 cfu/g in HES group, and 1.29 mg and $5.9x10^5$ cfu/g respectively, in MTX plus HES-Group *(Table 1)*.

There was no growth in mesenteric lymph node specimens in either control or HES groups. In other words, no bacterial translocation was seen in the intestine. Although the bacterial load of ascending colon was similar to the control and HES groups, significantly fewer bacteria grew in the ileocecal region in HES group. Serious translocation was seen in MTX group in the mesenteric lymph node specimens. However translocation was also seen in the group given MTX plus HES-Group but translocated bacteria density was 10 times lower than that of MTX group (P= 0.028). In other words, HES reduced but did not completely prevent the mucosal damage caused by MTX *(Table 1)*.

Ileocecal tissue specimens showed that MTX significantly increased the bacterial load comparing to MTX plus HES-Group (P=0.009, P=0.009). In the ascending colon, the bacterial load was higher, up to Log10, in the MTX group than that of HES and MTX plus HES-Group. The comprehensive information about the mean weight and bacteria densities in groups lymph node specimens was presented in *Table 1*.

DISCUSSION

Intestinal mucositis remains a major concern during cancer chemotherapy in more than 40% of cancer patients after standard doses of treatment, and in almost 100% of patients treated with high doses. In the gut, mucosal damage and barrier function alterations have been described as consequences of different processes: apoptosis, hypo-proliferation, inflammatory response, altered absorptive capacity, and bacteria proliferation and colonization [12,13].

Bacterial translocation is the passage of viable indigenous bacteria from the gastrointestinal tract to extra-intestinal sites. These include the mesenteric-lymph-node complex, liver, spleen and bloodstream. Three major mechanisms are involved in bacterial translocation: excessive intestinal bacterial growth, deficiencies in host immune defenses and increased intestinal mucosal barrier permeability or damage [14,15].

Recent research has concentrated on the role of the gastrointestinal tract as a reservoir for pathogens that can translocate to the circulation, initiating the septic process and eventually resulting in multiple organ failure. Until recently, most experimental studies have been performed in animal models and have quantified translocation by the recovery of viable micro-organisms from the mesenteric lymph nodes and other tissue by means of culture techniques [16]. Bacterial translocation has been reported in cases of burns [17], hemorrhagic shock [18], endotoxemia [19] and Crohn's disease [20]. The impairment of intestinal barrier functions caused by chemotherapy has been investigated in some studies, but there is a lack of research providing definitive evidence of chemotherapy-associated bacterial translocation [1,21-24].

Table 1. *Mean weight and bacteria densities in groups lymph node specimens*
Tablo 1. *Grupların lenf nodu örneklerinin ortalama ağırlık ve bakteri yoğunlukları*

Specimen	Control (n: 10)		MTX (n: 10)		HES (n: 10)		MTX plus HES (n: 10)		p1	p2	p3
	mg Mean (min-max)	cfu/g Mean (min-max)	mg Mean (min-max)	cfu/g Mean (min-max)	mg Mean (min-max)	cfu/g Mean (min-max)	mg Mean (min-max)	cfu/g Mean (min-max)			
Mesenteric-lymph-node	0.46 (0.34-0.60)	0.0 (0.0-0.0)	0.41 (0.35-0.48)	$4.0x10^3$ ($1.8x10^3$-$5.5x10^3$)	0.42 (0.34-0.50)	0.0 (0.0-0.0)	0.38 (0.29-0.45)	$9.1x10^2$ (0.0-$2.3x10^3$)	0.005	0.054	0.028
Ileocecal region	0.24 (0.18-0.31)	$4.0 x 10^5$ ($4.0x10^4$-$4.3x10^5$)	0.41 (0.35-0.48)	$5.0x10^6$ ($4.3x10^6$-$5.5x10^6$)	0.42 (0.34-0.50)	$4.0x10^3$ ($3.3x10^3$-$4.4x10^3$)	0.38 (0.29-0.45)	$8.0x10^4$ ($7.0x10^4$-$9.2x10^4$)	0.009	0.12	0.009
Ascending colon region	1.34 (0.88-1.82)	$5 x 10^5$ ($4.6x10^5$-$5.5x10^5$)	1.13 (0.90-1.35)	$5.0x10^6$ ($4.5x10^6$-$5.8x10^6$)	0.92 (0.55-1.18)	$2.0x10^5$ ($1.6x10^5$-$2.7x10^5$)	0.95 (0.72-1.29)	$5.5x10^5$ ($5.2x10^5$-$5.9x10^5$)	0.009	0.046	0.009

p1: *Comparison of control group and MTX group bacterial density,* ***p2:*** *Comparison of control group and MTX plus HES group bacterial density,* ***p3:*** *Comparison of MTX group and MTX plus HES group bacterial density*

HES enhances epidermal permeability barrier homeostasis. This is at least partly the result of stimulation of epidermal proliferation and differentiation [25]. Xu et al.[26] showed that HES is effective in an experimental study in which they induced experimental colitis with dextran sulphate. We investigated the effect of HES on MTX-induced bacterial translocation in rats. Oral administration of HES significantly decreased bacterial translocation. These results demonstrate that HES could ameliorate MTX-induced intestinal epithelial damage, our knowledge our study is the first study that MTX-induced bacterial translocation could be ameliorated by HES treatment in the intestine. The culture techniques used in this study were performed to confirm whether intestinal bacteria can translocate to extraintestinal organs such as the MLNs, ileocecal region and ascending colon region in a rat model of chemotherapy. One recent study showed that MTX induced severe damage of small intestinal mucosa and barrier functions in rats. Granulocyte colony-stimulating factor (G-CSF) given subcutaneously to rats is significantly effective in the preventing of bacterial translocation and morphological distortion after complete mechanical intestinal obstruction [3].

There was no growth in mesenteric lymph node specimens in the control and HES groups in this study; in other words, no bacterial translocation occurred. At the same time, while the two groups were similar in terms of ascending colon bacterial loads, significantly few bacteria grew in the ileocecal region in rats belonging to the HES group. Serious translocation was observed in the MTX group, and while translocation was seen in the MTX plus HES group rats, the translocated bacterial load was 10 times less that in the MTX group. In conclusion, our data suggested that HES could ameliorate MTX-induced bacterial translocation. However, it is difficult to explain the possible mechanism of HES based on the present data. Further studies are therefore needed to clarify the exact mechanism.

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