Evaluation of the Effects of Chitosan on Methotrexate-Induced Oral Mucositis in Rats

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Abstract

- I. Background: Methotrexate (MTX), a chemotherapeutic agent, is known to cause oral mucositis. Chitosan has been shown to have a protective effect in inflammatory animal models. This research aimed to examine the protective effect of chitosan against oral mucositis caused by MTX.
- II. Methods and Results: Wistar albino rats were randomly divided into three groups, 8 in each group as follow: Control (saline via oral gavage for 5 days), MTX (60 mg/kg single dose MTX intraperitoneally on 1st day and for following 4 days saline via oral gavage), and MTX+Chitosan(1st day single dose 60 mg/kg MTX intraperitoneally and followed with 200 mg/kg Chitosan via oral gavage for 4 days). After 24 hours of the last dose, animals were euthanised. Blood, tongue, buccal and palatal mucosa tissues were collected. Serum interleukin 1-beta (IL1- β), tumour necrosis factor-alpha (TNF- α), matrix metalloproteinase (MMP-1, and MMP-2) activities, and tissue bcl-2/bax ratio and the expression of caspase-3 (casp-3), and casp-9 were detected. The tissues were also examined histologically. Serum TNF- α , IL1- β , MMP-1 and MMP-2 activities and tissue casp-3 and casp-9 activities significantly increased but the bcl-2/bax ratio significantly decreased in the MTX group compared to the control group. Histologically, diffuse inflammatory cells were observed in MTX group. However, In the MTX + Chitosan group, all parameters approached the values of control group.
- III. Conclusion: Chitosan has been found to have a protective effect against oral mucosal damage caused by MTX. Thus, it may be a candidate agent against MTX induced oral mucositis.

1. Introduction

In cancer treatment, chemotherapy drugs play central roles, which have significant side effects on proliferative tissues. Radiotherapy combined with chemotherapy is another factor affecting dividing cells [1]. These patients who receive dual therapy are among the high-risk groups in terms of toxicity [2, 3]. One of the most common and devastating side effects observed with these treatments is oral mucositis. Oral mucositis leads to atrophy and ulceration with potentially serious complications in oral mucosa [4–6]. Severe oral mucositis, which occurs as a treatment complication, causes an interruption in anticancer treatments and significantly decreases patients' quality of life. Therefore, it is crucial to prevent or promptly treat oral mucositis symptoms [7, 8]. Various anti-inflammatory drugs and mouthwashes are using to treat oral mucositis, but it has reported that there is not enough therapeutic effect [9, 10]. Studies on oral mucositis have focused on the mechanisms involved in the generation of toxicity and highlighted apoptosis genes' key roles [11–14]. The ultimate purposes of mucositis physiopathology studies were to reduce or even prevent mucositis formation and determine the effect of radical therapy on target mechanisms [14–16].

In this study, Methotrexate (MTX) was used to induce oral mucositis. It is an antiproliferative folic acid analogue, an inhibitor of dihydrofolate reductase and an agent used in various chronic inflammatory diseases and cancer treatment [17]. In inflammatory conditions, low doses use, whereas high doses in

malignancies. High MTX doses cause serious side effects. MTX damages the oral tissues, primarily by destroying the mucous layer. MTX destroys rapidly dividing cells in the body, such as the gastrointestinal epithelium, including the buccal epithelium [6]. The use of MTX mainly causes oral mucositis with a rate of 40% in cancer chemotherapy. Pro-inflammatory cytokines and reactive oxygen species play an essential role in forming mucositis [18–21].

Apoptosis plays an essential role in many physiological processes, including pathologies in the mucosa [22]. Although the distinctive feature of many degenerative disorders is excessive apoptosis, reduced or absent apoptosis observe in some proliferative diseases [23]. It has known that MTX generally damages the proliferation of basal epithelial cells in the oral mucosa and cause oral mucositis in cancer patients [24]. Matrix metalloproteinases (MMPs) act as modulators in both regulating the homeostasis of the extracellular matrix and the response to inflammation and tissue damage during mucositis development. They have essential enzyme roles in mucosal physiology. Previous studies have reported that these enzymes participate in the ulcerative phase of oral mucositis by disrupting normal cell kinetics. They cause mucosal pathologies by mediating apoptosis [25, 26].

Chitosan is a degradable and non-toxic biopolymer obtained by deacetylation of chitin and used to treat oral mucositis. It is superior to other biopolymers due to its properties. Its antioxidant properties have reported in previous studies. Also, it has been shown in the literature that chitosan prevents tissue damage with its antioxidant and anti-inflammatory properties [27–29]. Moreover, it is non-toxic and biodegradable that makes chitosan superior to other biopolymers [29, 30]. Because of these significant effects of chitosan, we planned to investigate the protective effects of Chitosan against to MTX induced oral mucositis in the presented study.

Rats, medium-sized, long-tailed rodents, are ordinarily utilized in animal experiments of tissue injuries that resulted from inflammation as they are very similar to human beings genetically [31, 32]. In this study, the response of the methotrexate-induced mucositis to chitosan treatment was investigated using a rat model. This study aimed to examine and demonstrate the alterations in systemic and tissue levels caused by chitosan treatment and its healing effects biochemically, immunohistochemically and histopathologically.

2. Material And Method

2.1. Animals

The animals were obtained from the Near East University Animal Experiments Unit. The rats were maintained under a 12:12 h dark/light cycle from the start at a temperature of 22 ± 2 ° C in the humidity-controlled room ($50 \pm 5\%$) under ad libitum feeding without any restriction on their standard rat chow and water. The rats were housed in each plexiglass cage (4 rats/cage, 60×40×40 cm). Ethical approval of research was obtained from the Near East University Local Animal Experiments Ethics Committee on 17/04/2020 / no: 2020/111.

2.3. Experimental design and induction of experimental oral mucositis

The study was randomized, controlled, single-centre, single-blind (the study analyst was blind to the medications administered to the rats), and gender selected homogeneously (i.e., four males and four female rats for each group). In the study, 24 Wistar Albino rats of both sexes used, weighing 200-250 g, eight rats in each group randomly divided into three groups. 1. group (control, n=8): no procedure applied to the animals, only saline solution was administered by oral gavage for 5 days. 2. group (MTX, n=8): MTX was administered single dose (60 mg/kg) intraperitoneally on 1st day and for following 4 days, saline solution was administered[20]. 3. group (MTX + Chitosan, n=8): 1st day single dose (60 mg/kg) MTX was administered intraperitoneally and followed with Chitosan (200 mg/kg) administration by oral gavage for 4 days [27]. All animals observed for 5 days.

Twenty-four hours after the last injection, all rats were euthanized by decapitation. After killing all animals, blood samples were collected into serum separator tubes and delivered to Diagnostic Laboratory of Animal Hospital, Near East University. Sera were separated at 1500g x 10 minutes and stored at -80° C until analyses. Tongue, buccal and palatal mucosa tissues were extracted. Interleukin 1-beta (IL1- β), tumour necrosis factor-alpha (TNF- α), matrix metalloproteinase (MMP-1, and MMP-2) activities were analyzed spectrophotometrically (Mindray, BS120 automated biochemistry analyzer) in the serum for biochemical analysis. The bcl-2/bax ratio, the expression of caspase-3 (casp-3), and casp-9 were analyzed in all tissues by the western blotting method and examined histologically.

2.4. Biochemical Analysis

TNF- α , IL-1 β , MMP-1 and MMP-2 were measured on serum samples following the manufacturer's instructions and guidelines using commercially available rat specific enzyme- linked immunosorbent assay (ELISA) kits (ELR-TNF- α -2; ELR-IL1 β -2; ELR-MMP1-2; ELR-MMP2-2, RayBiotech Inc., GA 30092, USA). The washing steps were performed using an automated microtiter washer (MW-12A Microplate washer, Mindray, Shenzhen, China). The samples' optical density was obtained using a microtiter plate reader (MR-96A Microplate reader, Mindray), and results were calculated according to instructions.

2.6. Western blotting

After killing the rat, all tissues (tongue, palatal and buccal mucosa) were dissected, frozen, and stored at -80° C. Extracted tissues of four animals from each group homogenized and 10 min. centrifuged at 2000 G, lastly incubated with 0.1 mM EDTA, 0.5 mM DTT, % glycerol, 0.0 % Triton X-100, protease inhibitors and 10 mM Tris-HCl for 1hour. The amount of protein in each tissue determined by Lowry method [33]. 100 μ g protein was used for each tissue to prepare samples and loaded on gel electrophoresis (12% SDS-PAGE gel). Then samples were put into the nitrocellulose membranes (Schleicher and Schuell, 0.45 m, Germany) for 75 minutes at 80 V. The incubation of all membranes done using Alkaline phosphatase-conjugated rabbit monoclonal anti-goat IgG as the secondary antibodies for 1hour. After, 16 hours' incubation with primary antibodies [bax (1:100; sc-20067), bcl-2 (1:200; sc-7382), casp-3 (1:200; sc-

56053), and casp-9 (1:200; sc-56076) at +4 ° C, β -actin (1:100; sc-130657) was used for standardization in all membranes. Finally, the Bio-Rad Molecular Analyst Software used for the densitometric analysis of the membranes (www.totallab.com, Free). Molecular masses for bax, bcl-2, casp-3, casp-9 and β -actin are 23, 26, 35, 20, and 43kDa, respectively.

2.7. Histological examination and light microscopic analysis

Hematoxylin and Eosin (H&E) staining Paraffin wax blocks of the fixed tongue, palatal and buccal mucosa tissues were sliced as 4 μ m sections by using a rotary microtome and H&E used for histological staining. Olympus Microscope (BH-6, Shinjuku, Tokyo, Japan) used for histological inspection of H&E stained sections. Micrographs set at 200 $\times$ magnification (Olympus Altra 20). The epithelial thickness of the oral mucosa was measured using xxx software. An average of 15 to 20 measurements was recorded between the surface and basal layers of the epithelium's chosen piece.

2.8. Statistical analysis

Statistical analyses were performed using GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA). Quantitative data presented as mean $\pm$ standard error of mean (SEM) for eight animals in each group. To compare differences between more than two independent variables, one-way analysis of variance (ANOVA) was employed and followed for determining the differences between multiple groups; Tukey's multiple comparison tests were used. A value of $p \leq 0.05$ was taken to indicate statistical significance.

3. Results

3.1. Serum Levels of TNF- α , IL-1 β , MMP-1 and MMP-2.

Serum levels of pro-inflammatory cytokines (TNF- α , IL-1 β) and matrix metalloproteinases, (MMP-1, and MMP-2) were significantly higher in the group 1 (MTX-induced oral mucositis) than the control group in all tissues ($p < 0.001$). On the other hand, it determined that the increase in group 2 (MTX) almost returned to control values with Chitosan treatment (group 3, MTX + Chitosan), ($p < 0.05$ - 0.001 ; Fig. 1).

3.2. Western blotting analyzes

Western blotting experiments performed to investigate the effect of chitosan treatment on the bax, bcl-2, casp-3, and casp-9 protein levels in the tongue, palatal and buccal mucosa tissues of MTX-induced oral mucositis of rats ($n=8$ for each group). The membranes and representative western blottings of the oral tissues were illustrated in figure 2 (all membranes normalized by using a β -actin antibody).

The expression level of bax was statistically significantly increased in the MTX group than the control group in all examined tissues. The expression level of bax was substantially higher in the MTX group than the chitosan-treated group. Conversely, in the MTX group, the levels of bcl-2 were significantly lesser

compared to the control and MTX + Chitosan groups. Moreover, expression levels were similar in all tissues between the control and MTX + Chitosan groups. In the MTX group, the bcl-2/bax ratio was significantly lower compared to the control ($p<0.0001$, for all tissues) and the MTX + Chitosan groups ($p<0.01-0.001$; Fig. 3a-3c).

When the casp-9 expression analyzed in all regions determined that the casp-9 expression levels of MTX group rats increased compared to the control group ($p<0.05$, for all tissues), however, in group 3, the chitosan treatment caused the casp-9 expression levels to increase compared to the MTX group in all tissues ($p<0.05$; Fig. 3d-3f).

The casp-3 expressions were markedly elevated in the MTX group in all regions compared to the control group ($p<0.05-0.01$). MTX + Chitosan significantly decreased casp-3 expression levels compared to the MTX group in all regions ($p<0.01-0.001$; Fig. 3g-3i).

3.3. Histological Analyses

Tongue tissue: Inflammatory cells were not observed in submucosa (control group), moderate inflammatory cells were found in submucosa (MTX group) and rare inflammatory cell was found in submucosa (MTX + Chitosan group), (Fig. 4a, 4b, 4c, respectively).

Palatal tissue: Inflammatory cells were not detected in submucosa (control group), inflammatory cells were found in submucosa (MTX group), rare inflammatory cell was found in submucosa (MTX + Chitosan group), (Fig. 5a, 5b, 5c, respectively).

Buccal tissue: Inflammatory cells were not observed in submucosa (control group), moderate inflammatory cells were seen in submucosa and lymphoid aggregate was found in muscular layer (MTX group), rare inflammatory cell was observed in submucosa (MTX + Chitosan group), (Fig. 6a, 6b, 6c, respectively).

4. Discussion

Chemotherapy and radiotherapy cause damage to the mucosal surface, resulting in difficulty eating, drinking, and swallowing [34]. In this study, the effect of chitosan on oral mucositis caused by MTX evaluated by taking the tongue, palatal and buccal tissues of rats. Inflammation assessed by examining the cytokine (IL-1 β and TNF- α) expression and MMPs (MMP-1, MMP-2) in blood and the levels of bcl-2 / bax ratio, caspase-3, caspase-9 in the tissues at the end of the experiment. Furthermore, structural damage was evaluated by histological examinations.

In this study, we found that blood IL-1 β and TNF- α levels increased with MTX administration to the rats, and these cytokine levels decreased to control levels with chitosan administration. This result is compatible with the literature and evaluated due to the development of mucositis with chemotherapy [35]. It has shown that with the application of MTX, the increase in IL-1 β and TNF- α levels in the blood plays a role in the mechanism of damage in different tissues [36]. It has also shown that chitosan

reduces increased levels of IL-1 β and TNF- α in inflammation models in rats [37]. Both our results and the findings of the literature suggest that chitosan has anti-inflammatory properties and thus may be effective in oral mucositis damage.

MMPs regulate apoptotic and anti-apoptotic actions in cellular death in an intriguing way [38]. For example, MMP-2 separates stromal cell-derived factor- α and cause neural death of the cell. Further, the apoptosis of basal keratinocytes of the oral mucosa is associated with MMPs. Since it thought to upregulate the factors involved in apoptosis [22]. Previous in vivo studies showed that the lack of epithelial cells in the underlying basement membrane and subsequently lack of the source of MMPs, upregulation of apoptosis-related genes and finally, death of cell documented [39]

Moreover, in cancer studies, the anti-apoptotic activity of MMPs have also determined by stimulating tumour invasion and metastasis. Therefore, it secures apoptosis of the invading tumour cells [40]. MMPs changes the micro-environment and alter cellular behaviour by affecting ECM molecules, including morphogenesis and cell proliferation [41]. MMPs and their inhibitors also occur in such oral mucosal disorders as oral lichen planus and aphthous ulceration [42, 43]. Besides, some gastrointestinal diseases may cause symptoms in the oral mucosa; therefore, it is logical to speculate common pathophysiology in both mucosas. Mutual pathophysiological processes of autoimmune diseases, such as pemphigus, pemphigoid, and systemic lupus erythematosus, should be considered while investigating some inflammatory mediated skin diseases which affect the oral mucosa [44]. Additionally, in Stevens-Johnson syndrome, hypersensitivity reaction lesions occur, and toxic epidermal necrolysis may widely participate in the oral mucosa [45].

MMPs are related to the pathophysiology of these conditions. In our study, it has shown that serum MMP-1 and MMP-2 levels increased with methotrexate administration. These results are consistent with the literature. MMP-1 and MMP-2 levels approached control values with Chitosan application. The effects of chitosan on MMP-1 and MMP-2 expressions have studied in various models. In these models, chitosan has a protective effect with its anti-inflammatory effects. However, the impact of chitosan on oral mucositis has not studied before. With this study we have done, chitosan will prevent oral mucositis that may occur during MTX treatment.

Mitochondria play a significant role in apoptosis. MTX is known to induce apoptosis along with mitochondrial dysfunction. There is a balance between anti-apoptotic and proapoptotic genes [46]. While Bax causes the activation of apoptosis, the bcl-2 protein exerts an anti-apoptotic effect by preventing Bax activation. Caspases act as the primary applicators of apoptosis. The apoptosis in methotrexate-induced oral mucositis and determination of apoptosis by using DNA fragmentation observed in only one study[19].

DNA fragmentation is an indicator of apoptosis and its necrosis and is not a suitable marker for apoptosis. Our study demonstrated the importance of apoptosis in mucositis by evaluating the bcl-2 / bax ratio and caspase activation. It has shown in the literature that chitosan used in this study prevents

apoptosis in addition to its anti-inflammatory effect. These results are consistent with the results of the present research.

In this study, increased inflammatory cells were detected in all tissues in the methotrexate group while the researchers evaluated histopathological examinations. A structural improvement observed with chitosan. Studies have shown that damage occurs to the mucosa due to chemotherapeutic agents inducing cytokine levels and the apoptotic process [35, 47]. Our study observed that chitosan contributes to tissue healing thanks to its anti-inflammatory properties, following the literature.

As a result, participating researchers in this study found that chitosan exerts a protective effect by suppressing the cytokine expression and apoptotic processes in oral mucositis formed in fornix, tongue, and palate tissues by methotrexate administration. The use of this non-cytotoxic agent together with methotrexate is vital in terms of reducing its toxic effects.

Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Author Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Kani Bilginaylar, Asli Aykac, Serkan Sayiner, Hanife Ozkayalar and Ahmet Ozer Şehirli. The first draft of the manuscript was written by Kani Bilginaylar and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Near East University Local Animal Experiments Ethics Committee on 17/04/2020 / no: 2020/111.

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Figures

Figure 1

The levels of serum a) TNF-α, b) IL-1β c) MMP-1 and d) MMP-2 of tongue, palatal, and buccal tissues in the control, MTX, and MTX + Chitosan groups. ***p<0.001 Comparisons according to control group, +++ p<0.001 Comparisons according to MTX group.

Figure 2

The representative images of nitrocellulose membranes obtained from immunoblotting experiments of the tongue, palatal, and buccal tissues in control, MTX, and chitosan-treated groups protein expressions of bax, bcl 2, β -actin, casp-3, and casp-9.

Figure 3

The expression levels of bcl-2 / bax ratio, casp-3 and casp-9 of tongue, palatal, and buccal tissues in the control, MTX, and MTX + Chitosan groups. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ Comparisons according to control group, ++ $p < 0.01$ and +++ $p < 0.001$ Comparisons according to MTX group.

Figure 4

Histological view of tongue tissue a) Control group b) MTX group c) MTX + Chitosan group, Magnification X200.

Figure 5

Histological view of palatal tissue a) Control group b) MTX group C) MTX + Chitosan group, Magnification X200.

Figure 6

Histological view of buccal tissue a) Control group b) MTX group c) MTX + Chitosan group, Magnification X200.