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EFFECT OF GREEN TEA AND BLACK SEED ON METHOTREXATE INDUCED CYTOTOXICITY OF THE ORAL MUCOSA, TONGUE AND THE SUBMANDIBULAR SALIVARY GLAND OF ALBINO RATS

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ABSTRACT

Background: Chemotherapy is an important intervention for treatment of cancer. Methotrexate (MTX) is a drug which has a wide application in the treatment of malignant diseases and autoimmune diseases. The main side effect of MTX is oral mucositis as well as salivary gland damage. Oral mucositis is a critical complication causing discontinuing of chemotherapy with a negative impact on diet, oral hygiene and quality of life. **Objective:** to evaluate and compare the individual effect of green tea and black seeds oil extract, on MTX induced oral mucositis and submandibular salivary gland cytotoxicity in albino rats.

Material and Methods: A total of fifty six male Albino rats were used. The rats were divided into four experimental groups, Group 1 (G1: control group), Group 2 (G2: MTX treated group), Group 3 (G3: MTX + Green Tea extract treated group) and Group 4 (G4: MTX + Black seed oil treated group). Rats were sacrificed separately after five and ten days; then immediately dissected to obtain the submandibular salivary gland, a biopsy from the buccal mucosa and another from the tongue mucosa. Specimens were processed for hematoxylin and eosin staining and immunohistochemically prepared for COX-2 expression. The area fraction of COX-2 expression was calculated. The data was analyzed and expressed statistically.

Results: Group 1 showed normal structure of the salivary gland, the oral and tongue mucosa. Group 2 treated with MTX showed severe loss in the normal histologic structure of the fore mentioned tissues with marked inflammatory cell infiltration. Group 3 showed almost normal epithelial lining of both the gland and the mucosa with reduction in the inflammatory cell infiltrate. Group 4 also showed reduction in signs of inflammation with areas of fibrosis. Immunohistochemical results revealed a weak positive reaction of COX 2 in groups 1, 3 and 4 while a strong positive reaction was noticed in group 2.

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Conclusion: The present study revealed that MTX caused severe degenerative changes in the histologic structure of the submandibular gland with increased fibrosis and inflammatory cell infiltration. The green tea caused marked improvement in the architecture of the ducts with almost normal lumen and epithelial lining, with obvious anti-inflammatory effect after five days of treatment. Further improvement was noticed after ten days. Black seed had a cytoprotective effect on the parenchymal cells of the gland and the oral mucosa as well as anti-inflammatory and antioxidant benefits.

KEYWORDS: MTX, salivary glands, green tea, black seed, COX-2

INTRODUCTION

Methotrexate (MTX) has long been used as a chemotherapeutic drug for treatment of variable types of malignancies as acute leukemia and osteosarcoma as well as several autoimmune diseases as rheumatoid arthritis, lichen planus and psoriasis [1, 2]. The mechanism of action of MTX was found to depend mainly on the suppression of proliferation of these abnormal cells, by inhibition of DNA synthesis [3].

Methotrexate is a synthetic organic compound. It belongs to an anti-folate therapeutic group and to class III of the Biopharmaceutical Classification System. It was first introduced in the late 1940s as a less toxic derivative of aminopterin and then it was synthesized as a folic acid antagonist which was later used to treat children with acute leukemia [3, 4]. Later in the early 80s, researchers noted that the use of a low dose of aminopterin (1–2 mg/day) leads to a significant improvement in patients with rheumatoid arthritis and others with psoriasis [5]. Nowadays, MTX is commonly utilized in combination with other drugs as a combined chemotherapy for the treatment of many neoplasms as acute leukemia, non-Hodgkin's lymphoma and breast cancers [6], severe and resistant forms of autoimmune diseases of rheumatoid arthritis, psoriasis and Crohn's disease [7-10]. MTX is commonly used as an immunosuppressive drug in treatment of patients with Crohn's disease. It is usually used in combination with anti-tumor necrosis factor (TNF) agents to inhibit immunogenicity [11-13].

However, when MTX is used in high doses in cancer therapy in combination with other therapeutic drugs, it may cause very serious, life-threatening side effects, which is usually dependent on the treatment duration, age and condition of the patient. Generally, during oncological therapy with MTX, the adverse reaction effects happen in more than 1 to 10 people (>10%) in a dose and duration dependent manner [14]. MTX administered for a long time, causes a gradual decrease in the number of blood cells with decrease in the activity of the immune system, respiratory failure and liver diseases. Other manifestations include serious skin reactions bone marrow suppression as well as severe damage to the salivary glands and oral mucositis which could contribute to high rate of morbidity and mortality in cancer patients [15-17].

Oral mucositis is one of the main and serious complications resulting from chemotherapy. Chemotherapy kills epithelial cells of mucosa causing direct damage or atrophy which could be in the form of diffuse or localized ulceration. MTX has a cytotoxic effect on the epithelial cell layer which causes destruction of DNA of progenitor cells belonging to the basal cell layer and induces apoptosis and dysfunction of these cells [18]. These lesions may appear as ulcerative painful lesions which markedly affect diet and oral hygiene and have a negative influence on the patient's life. In immunocompromised patients, these ulcerative lesions can be secondarily infected and lead to spread of infection and sepsis [19].

Moreover, the oral micro-flora plays a role in the progression of oral mucositis. Administration of chemotherapy leads to an alteration in the ecological balance. The damage in the oral mucosa and the depletion of neutrophils resulting from chemotherapy, allow some of the resident flora to shift to pathogenesis causing an exacerbation of the inflammatory process and leading to bacteremia in immunocompromised patients [20].

Furthermore, MTX induced cytotoxicity in salivary glands appears as destruction and damage of the histological structure of the acini, dilatation of the ducts as well as alteration in the composition of the salivary secretion, which results in hyposalivation and xerostomia, finally leading to oral mucositis and its complications [21]. Many factors are responsible for that including the direct inhibitory effect of MTX on cell division in the acini and ducts [21]. A previous study revealed that the concentration of S-IgA, IgG and IgM in stimulated saliva was markedly reduced during treatment with chemotherapeutic agents [22].

Different agents are used to treat oral mucositis, ranging from pharmacological drugs and mechanical stimuli to palliative treatment like saliva substitutes, chewing gum, mouth washes and oral gels [23]. The use of natural compounds in treatment of diseases is attractive as they are supposed to have fewer side effects when compared to synthetic drugs. These compounds play an important role in prevention and decreasing the incidence of oral mucositis. Among these natural compounds are Green Tea and Black Seed [24, 25].

The main chemical constitute of tea is catechin, which has antioxidant as well as antiapoptotic, anti-inflammatory and antiangiogenic properties. It also contains carotenoids, vitamin E, tocopherols, caffeine, vitamin C, minerals such as zinc, chromium, manganese, and certain phytochemical compounds which are able to reduce severity of oral mucositis [24, 26].

Black and oolong tea do not give the same benefits as Green and white tea [48]. EGCG is also found in Green Tea with higher amounts than those in oolong tea and black tea, which justify the superiority of Green Tea in health benefits [24].

The antioxidant role of Green Tea is associated with essential nutrient vitamin E, which has a protective role against the damaging effect of free radicals. Vitamin C, which is one of the nutrients found in Green Tea, in combination with vitamin E, both act on the cellular level protecting the cell membrane and preventing peroxidation [27]. Vitamin C exhibits antiscorbutic activity, prevents cataracts and reinforces the immune system. It was proved that vitamin E can be used as a treatment for oral mucositis. Furthermore, Green Tea has antiviral, antibacterial and antifungal activity especially against Candida Albicans [28, 29]. Benefits of Green Tea against the progression of precancerous lesions toward malignant transformation in the oral cavity were also reported [30].

Black seed has several therapeutic benefits, such as anti-cancer, antimicrobial, analgesic, antipyretic, anti-tussive, anti-inflammatory and antioxidant potentials. Black seed's antimicrobial effects include those on gram negative and gram-positive bacteria, parasites, viruses and fungi [31].

Thymoquinone (TQ), as an active constituent of Black Seed, has free radical scavenging properties and inhibits lipid peroxidation [32]. Black Seed also is thought to have protective effect against chemotherapy induced toxicity which usually affects the quality of life and the nutritional state of the patient [33]. Besides the cytotoxic effect of essential oil and ethyl acetate extracts of Black Seed against various cancer cell lines, it was also associated to other mechanisms as: (a) promotion of tumor cell death and inhibition of its proliferation, (b) inhibition of tumor angiogenesis, invasion and metastasis [33].

Cyclooxygenases are enzymes that catalyze the synthesis of prostaglandins (PG) from arachidonic acid. There are two isoforms of COXs: COX-1 which is constitutively expressed in all mammalian tissues [34]. COX-1 is housekeeping enzyme that maintains homeostasis in most cells. The second isoform is COX-2. It is undetectable in most normal tissues, but can be rapidly induced by pathological conditions (inflammation and carcinogens). COX-2 stimulates production of PGs that mediate inflammation [35].

Cyclooxygenase-2 level is increased when a chemotherapy treatment is used, as chemotherapy can induce inflammation in tissues by stimulation of transcription factor nuclear factor kappa B (NFκB) which causes upregulation of COX-2 [36].

The present study was undertaken to evaluate and compare the effect of Green Tea and Black Seed oil on Methotrexate induced oral mucositis in the oral and tongue mucosa as well as submandibular salivary gland cytotoxicity in Albino rats.

MATERIAL AND METHODS

Fifty-six male albino rats, 10-12 weeks of age and weighing from 200 to 250 g, were obtained from the Animal Facility, Medical Research Center, Faculty of Medicine, Ain Shams University, Cairo, Egypt. They were housed in the separate cages, six rats per cage in the animal house of Faculty of Medicine, Ain Shams University, throughout the duration of the experiment.

The rats were kept under good ventilation and adequate stable diet consisting of fresh vegetables, dried bread and tap water throughout the experimental period (five and ten days). Diet and drinking water were given at liberty during the experiment. Incineration of sacrificed bodies was done in the incinerator at the animal house. All these procedures were reviewed by the Research Ethics Committee of the Faculty of Dentistry, Ain Shams University and were under the supervision of a specialized veterinarian.

The following materials were used:

1- Methotrexate vials purchased from Mylane SAS Corporation, France.

2- Green Tea extract was prepared at Faculty of Pharmacy, Ain Shams University.

3- Black Seed oil was purchased from Imtenan Health Shop, Cairo, Egypt.

Preparation of the extract of the Green Tea:

Daily method of tea making (household preparation) from Green Tea (Ahmed tea Brand 100% pure) was used to prepare aqueous extracts. The aqueous extract was prepared with the consideration of the absorption coefficient of Green Tea leaves (which is 2). Five grams of dried Green Tea leaves were grinded to pieces of diameter less than one mm, were poured with 60 ml of boiling distilled water and time was given for the extraction to cool down. The mixture was filtered first with a fine muslin cloth under pressure and the clear filtrate was dried in a water bath at 40°C until a paste was obtained, dissolved in distilled water and used at a concentration of 40mg/kg/day.

The experimental Design

The Albino rats were randomly divided into 4 groups, 14 rats in each group. Each group was subdivided into 2 subgroups; in the first subgroup the duration of the experiment was five days, and in the second subgroup the duration of the experiment was 10 days. Sacrifice of rats was done at the end of each duration.

The four groups were divided into groups and subgroups as shown in table 1.

Hematoxylin and Eosin Staining

The specimens taken at each time interval were

TABLE (1): Classification of groups and subgroups (S: submandibular salivary gland; M: for buccal mucosa; T: for dorsum of tongue; A: 5 days; B: 10 days).

	Submandibular salivary gland	Buccal Mucosa	Dorsum of tongue
G1 (Control Group)	Gl SA: control group salivary gland 5 days	G1 MA: control group buccal mucosa 5 days	Gl TA: control dorsum of tongue 5 days
	Gl SB: control group salivary gland 10 days	Gl MB: control group buccal mucosa 10 days	Gl TB: control dorsum of tongue 10 days
G2 (MTX treated Group)	G2 SA: MTX group salivary gland 5 days	G2 MA: MTX group buccal mucosa 5 days	G2 TA: MTX dorsum of tongue 5 days
	G2 SB: MTX group salivary gland 10 days	G2 MB: MTX group buccal mucosa 10 days	G2 TB: MTX dorsum of tongue 10 days
G3 (MTX + Green Tea treated Group)	G3 SA: MTX +Green Tea group salivary gland 5 days	G3MA: MTX + Green Tea group buccal mucosa 5 days	G3TA: MTX + Green Tea group dorsum of tongue 5 days
	G4SB: MTX + Black seed group salivary gland 10 days	G4 MB: MTX + Black seed group buccal mucosa 10 days	G4 TB: MTX + Black seed group dorsum of tongue 10 days

immediately fixed in 10% buffered formalin for 24 hours. Specimens were processed for hematoxylin and eosin (H and E) stain and then mounted with Canada balsam for histological examination by light microscopy.

Immunohistochemical examination

Paraffin embedded tissue sections were dewaxed and rehydrated through grade ethanol to distilled water. Endogenous peroxidase was blocked by incubation with 3% H_2O_2 in methanol for 10 minutes. The antigen retrieval was achieved by microwave in citrate buffer solution (pH 6.0) for 10 minutes, followed by washing step with Tris-buffered saline (pH 7.6). The tissue sections were then incubated with power Block TM reagent (Lab Vision, CA, USA), universal proteinaceous blocking reagent, for 15 minutes at room temperature to block non-specific binding sites. The tissue sections were then incubated with the primary anti-COX2 antibodies (cat# RB- 9072-P0) ready to use, purchased from Lab Vision, USA, overnight at 4°C. The bound primary antibody was detected by incubation with the secondary antibody conjugated with horseradish peroxidase (Lab Vision, CA, USA) for 30 minutes at room temperature.

After rinsing with Tris-buffered saline, the antigen-antibody complex was detected using 3, 3´-diaminobenzidine, the substrate of horseradish peroxidase. When acceptable color intensity was reached, the slides were washed, counter stained with hematoxylin and covered with a mounting medium. The slides were microscopically examined and analyzed by image analysis software (Image J 4.1).

Statistical analysis

The area fraction expressed by COX-2 was calculated histomorphometrically. Data were coded and entered using SPSS version 28 (IBM Corp., Armonk, NY, USA). Data was expressed as mean and standard deviation. Comparisons between quantitative variables were done using the non-parametric Kruskal-Wallis and Mann-Whitney tests [37]. P-values equal or less than 0.05 ($p \leq 0.05$) were considered as statistically significant.

RESULTS

Histologic results

a) Submandibular salivary gland

Control group (Gl)

Examination of H and E stained sections of submandibular salivary gland in Gl group showed in both subgroups G1SA and G1SB normal salivary tissue which consists of serous acini found in a well-arranged architecture as well as the granular convoluted tubules (GCT), intercalated (IC) and striated ducts (SD). They all showed normal lining of cuboidal epithelium, with prominent granular cytoplasm in the cells of the GCT. Minimal inflammatory cells were noted.

MTX treated group (G2)

G2SA subgroup showed a marked dilatation of the ducts with flattening of its lining ductal epithelium due to stasis of the acinar secretion within the ducts. Swelling of few acinar cells was noted. Inflammatory cells increased (Fig. 1). G2SB subgroup showed severe loss of acinar architecture, acinar degeneration and replacement with fibrosis was detected. Some acini showed variable nuclear size. Destruction of the ducts was also found and heavy inflammatory cell infiltrate was noted. Striated ducts showed shrunken cavities with vacuolation of the cells while GCT showed ill-defined outline with clumping of the cytoplasm and loss of granules.

MTX + Green Tea treated group (G3)

G3SA subgroup showed marked improvement in ductal cells. Almost normal epithelial lining was found. A reduction in inflammatory cell infiltrate and the fibrosis of the acini were noted (Fig.2). G3SB subgroup showed further improvement and restoration of structural integrity of the ducts and acini, with almost normal appearance of the granular cytoplasm of the cells of the GCT.

MTX + Black Seed treated group (G4)

G4SA subgroup showed that the ducts restored their integrity with reduction of signs of inflammation. Small areas of fibrosis were noted but markedly less than GS2A subgroup (Fig.3). G4SB subgroup revealed slight improvement in the structural integrity of the ducts and acini. Only a few GCTS still showed signs of degeneration, but generally the condition of the epithelium was much improved with less vacuolization and clumping of the cytoplasm. A few congested blood vessels could still be found.

b) Buccal mucosa

Control group (G l)

Examination of H and E stained sections of buccal mucosa in G1 group showed in both subgroups G1MA and G1MB normal histological features of the surface epithelium and underlying connective tissue. Minimal inflammatory cells were noted.

MTX treated group (G2)

G2MA subgroup showed increase in epithelial thickness of buccal mucosa (epithelial hyperplasia) and hyperkeratosis. Signs of inflammation were found in the connective tissue. Vacuolar degeneration of basal and prickle cell layer with basilar hyperplasia were noted (Fig. 2). In subgroup G2MB, epithelial hyperplasia, prominent rete pegs and inflammatory cell infiltration were found.

MTX + Green Tea treated group (G3)

G3MA subgroup showed a decrease of epithelial hyperplasia and keratin thickness with short, few and regular rete ridges. Vacuolar degeneration markedly decreased and minimal inflammatory cells were noted. Subgroup G3MB showed that the epithelium and keratin restored.

MTX + Black Seed treated group (G4)

G4MA subgroup presented improvement in its

histological structure. Minimal inflammatory cells were noted in connective tissue. G4MB also showed further improvement in the epithelium.

c) Dorsal surface of tongue

Control group (Gl)

Examination of H and E stained sections of dorsum of tongue in Gl group showed in both subgroups GITA and G1TB normal histological features of surface epithelium and underlying connective tissue. The connective tissue showed minimal inflammatory cell infiltration.

MTX treated group (G2)

G2TA subgroup showed epithelial hyperplasia and hyperkeratosis. The connective tissue showed dilated capillaries and inflammatory cell infiltration (Fig. 3). G2TB subgroup revealed epithelial hyperplasia and hyperkeratosis. Vacuolar degeneration in epithelial cells was noted with inflammatory cell infiltrate in the underlying connective tissue.

MTX + Green Tea treated group (G3)

G3TA subgroup showed decrease of epithelial hyperplasia and hyperkeratosis. Minimal inflammatory cells were seen. G3TB subgroup revealed further improvement in the histological structure and restoration of epithelial integrity.

MTX + Black Seed treated group (G4)

G4TA subgroup showed almost normal keratin and epithelial thickness. Subgroup G4TB showed also almost normal keratin and epithelial thickness (compared to G2TB).

B- Immunohistochemical Results

a) Submandibular salivary gland

Control group (Gl)

Examination of COX-2 stained sections of submandibular salivary gland of G1SA and G1SB subgroups revealed weak positive immunoexpression of the cytoplasm and nuclei of the ductal and acinar epithelium.

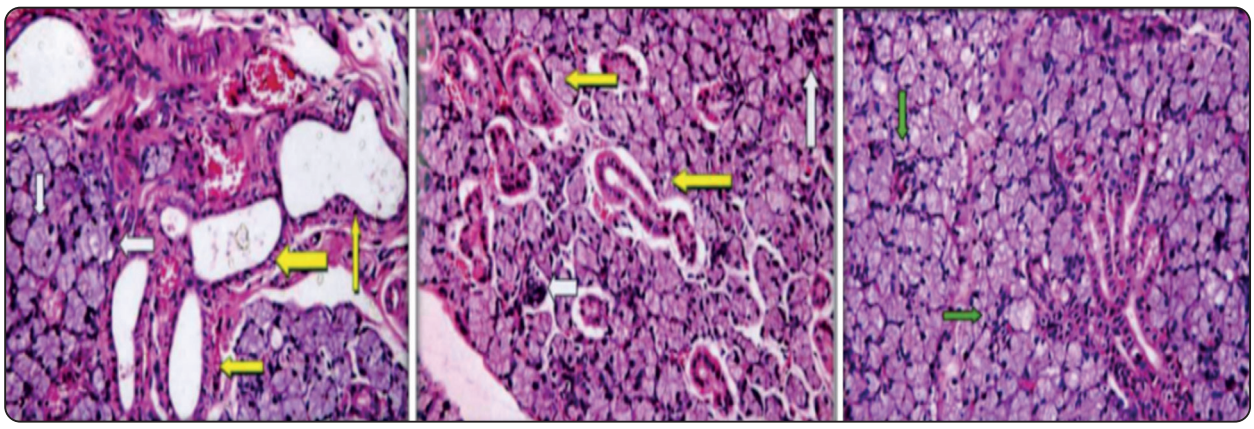

Fig. (1): A photomicrograph of G2SA showing dilatation of ducts with flattening of lining ductal epithelium (yellow arrows). Swelling of few acini is noted (white arrows) with congested blood vessels (H and E, original magnification X 40).

Fig. (2) A photomicrograph of G3SA showing improvement in structure of ducts with almost normal ductal lining cells (yellow arrows). Minimal inflammatory cells are noted (white arrows) (H and E, original magnification X 40).

Fig. (3) A photomicrograph of G4SA showing an improvement in ducts and acinic structure. Minimal inflammatory cells are noted (green arrows) (H and E, original magnification X 40).

MTX treated group (G2)

G2SA and G2SB subgroups revealed strong positive immunoexpression in the cytoplasm of the ductal epithelium. Positive reaction in acinar nuclei and cytoplasm was found in G2SA and absent in G2SB.

MTX + Green Tea treated group (G3)

G3SA subgroup showed positive immunoexpression in the cytoplasm of the ductal epithelium with weaker expression in the acinar cells. G3SB subgroup, however, revealed weak positive immunoexpression in the cytoplasm of the ductal epithelium with weaker expression in the acinar cells.

MTX + Black Seed treated group (G4)

G4SA subgroup showed positive immunoexpression in the cytoplasm of the ductal epithelium with weak acinar reaction. G4SB subgroup revealed weak positive immunoexpression in the cytoplasm of the ductal epithelium with no acinar reaction.

b) Buccal mucosa

Control group (Gl)

Examination of COX 2 stained sections of buccal mucosa of G1MA and G1MB subgroups revealed positively stained nuclei of basal epithelial cells and inflammatory cells.

MTX treated group (G2)

G2MA and G2MB subgroups revealed positively stained nuclei and cytoplasm of the epithelium, inflammatory cells and endothelial cells of blood capillaries (Fig.4).

MTX + Green Tea treated group (G3) and MTX + Black Seed treated group (G4)

G3MA and G4MA subgroup revealed positively stained nuclei and cytoplasm of basal and suprabasal cells of the epithelium. G3MB and G4MB subgroup revealed only some immunopositive basal epithelial cells and inflammatory cells (Fig.5).

c) Dorsal surface of tongue

Control group (Gl)

Examination of COX-2 stained sections of dorsal surface of tongue of GITA and G1TB subgroups showed immunopositive nuclei of epithelial basal cells and inflammatory cells.

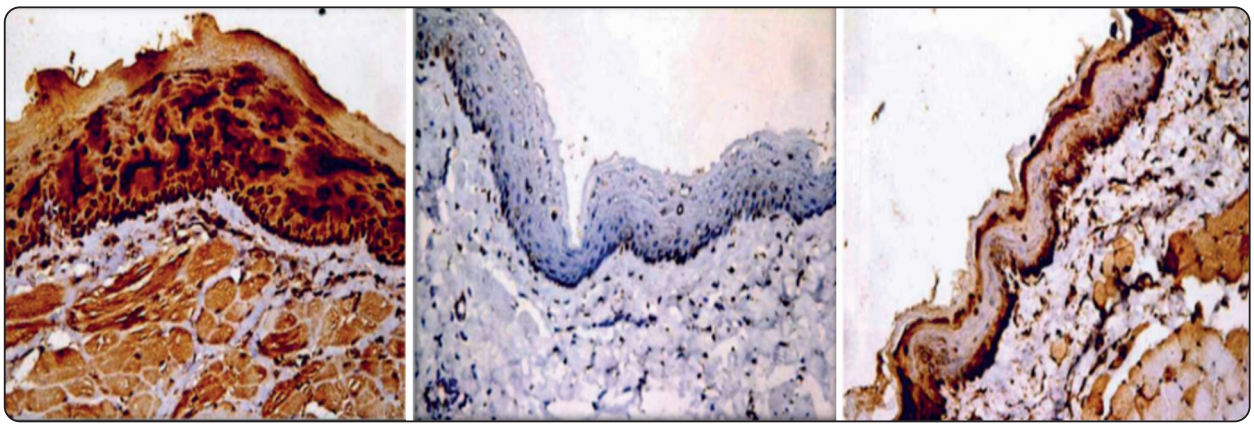

Fig. (4): A photomicrograph of G2MA showing positively stained nuclei and cytoplasm of the epithelium, inflammatory and endothelial cells of blood capillaries. (anti-COX 2, original magnification X 40)

Fig. (5): A photomicrograph of G3MB showing some immunopositive basal cells and inflammatory cells. (anti-COX. 2, original magnification X40).

Fig. (6): A photomicrograph of G4MB showing positively stained nuclei of epithelial basal cells and inflammatory cells (anti-COX 2, original magnification X 40).

MTX treated group (G2)

G2TA and G2TB subgroups revealed strong positively stained nuclei and cytoplasm of epithelium and inflammatory cells.

MTX + Green Tea treated group (G3) and MTX + Black Seed treated group (G4)

G3TA and G4TA subgroup revealed positively stained nuclei of epithelial cells with weak positively stained inflammatory cells. G3TB and G4TB subgroup also revealed weak positive nuclear and cytoplasmic reaction in the basal and prickle cells.

C) Statistical Results for Immunohistochemistry

a- Submandibular salivary gland:

The descriptive statistics for mean and standard deviation (SD) of mean area fraction (MAF) of COX-2 in all subgroups after 5 and 10 days duration are shown in table 2.

A significant statistical difference was detected between the four subgroups (P value = 0.005 and 0.019 respectively).

ANOVA , followed by Post hoc test for pairwise comparison, revealed a statistically significant difference between G2SA and G4SA (P = 0.008), G1SA and G2SA (P =0.001), G1SA and G3SA (P = 0.038), G2SB and G4SB (P =0.003) and also between G2SB and G3SB (P = 0.017), but no statistically significant difference was detected between G2SA and G3SA (P = 0.235), G3SA and G4SA (P = 0.137), G1SA and G4SA (P =0.552), G4SB and G3SB (P = 0.552), G1SB and G4SB (P = 0.234), G3SB and G1SB (P = 0.552) and also between G1SB and G2SB (P =0.074).

b- Buccal Mucosa:

The descriptive statistics for mean and SD of MAF of COX-2 in all subgroups after 5 and 10 days duration are shown in table 3.

TABLE (2): Descriptive statistics for the mean and SD of COX-2 in 4 subgroups after 5 and 10 days duration (submandibular salivary gland).

		Subgroup A				Subgroup B				P- value	
		G1SA	G2SA	G3SA	G4SA	G1SB	G2SB	G3SB	G4SB	Subgroup A	Subgroup B
Cox2	Mean	1.29^{a}	11.74^{b}	6.48^{bc}	2.72^{ac}	1.45^{ab}	9.4^{a}	1.5^{b}	1.25^{b}	0.005	0.019
	SD	0.39	0.89	1.94	1.67	0.1	1.72	0.68	1.37		

Different superscripts in the same subgroup mean statistically significant difference between the groups.
P value ≤ 0.05 is considered statistically significant.

TABLE (3): Descriptive statistics for the mean and SD of COX-2 in 4 subgroups after 5 and 10 days duration (buccal mucosa).

		Subgroup A				Subgroup B				P- value	
		G1MA	G2MA	G3MA	G4MA	G1MB	G2MB	G3MB	G4MB	Subgroup A	Subgroup B
Cox2	Mean	1.7^{a}	17.32^{b}	2.8^{a}	8.72^{ab}	1.6^{a}	9.5^{b}	1.33^{a}	2.78^{ab}	0.008	0.024
	SD	0.36	2.04	2.44	4.58	0.75	2.65	0.99	1.53		

Different superscripts in the same subgroup mean statistically significant difference between the groups.
P value ≤ 0.05 is considered statistically significant.

A significant statistical difference was detected between the four subgroups (P value = 0.008 and 0.024 respectively).

ANOVA, followed by Post hoc test for pairwise comparison, revealed a statistically significant difference between G2MA and G3MA (P =0.005), G1MA and G2MA (P =0.003), G2MB and G3MB (P =0.006) and also between G1MB and G2MB (P =0.010), but no statistically significant difference was detected between G1MA and G3MA (P = 0.882), G2MA and G4MA (P = 0.181), G1MA and G4MA (P =0.102), G3MA and G4MA (P =0.137), G1MB and G3MB (P = 0.853), G3MB and G4MB (P = 0.353), G1MB and G4MB (P =0.457) and also between G2MB and G4MB (P =0.069).

c- Dorsum of Tongue:

The descriptive statistics for mean and SD of MAF of COX-2 in all subgroups after 5 and 10 days duration are shown in table 4.

A significant statistical difference was detected between the four subgroups (P value = 0.026 and 0.022 respectively).

ANOVA, followed by Post hoc test for pairwise comparison, revealed a statistically significant difference between G2TA and G4TA (P =0.021), G3TA and G2TA (P = 0.049), G1TA and G2TA (P =0.004), G2TB and G3TB (P =0.005) and also between G1TB and G2TB (P = 0.012), but no statistically significant difference was detected between G1TA and G4TA (P = 0.557), G1TA and G3TA (P = 0.373), G3TA and G4TA (P =0.738), G1TB and G3TB (P = 0.766), G3TB and G4TB (P = 0.229), G1TB and G4TB (P = 0.458) and also between G2TB and G4TB (P =0.075).

DISCUSSION

Oral mucositis is an inflammatory, ulcerative process which is usually seen in patients receiving chemotherapy and radiation for cancer treatment. Approximately 40% of all patients receiving chemotherapy and 80% of patients receiving radiotherapy for head and neck tumors are affected with oral mucositis [38].

Methotrexate, which is a folic acid analogue, is used as a chemotherapeutic drug that binds to (DHFR), resulting in a decrease in the pool of reduced folic co-enzymes necessary for nucleic acid synthesis. MTX may negatively affect normal as well as cancer cells in the oral cavity [1]. Oral mucosal cells have a very high cell turnover rate. This rapid course of cell proliferation and constant epithelial replacement render the mucosa susceptible to the effects of cytotoxic drugs that affect rapidly proliferating cells [39].

Salivary glands are a useful tool of investigation for the study of pharmacological agents' effect on different tissues. Despite their low rate of division, the tissue of salivary gland loses its function regularly with significant reduction of saliva production

TABLE (4): Descriptive statistics for the mean and SD of COX-2 in 4 subgroups after 5 and 10 days duration (dorsum of tongue).

		Subgroup A				Subgroup B				P- value	
		G1TA	G2TA	G3TA	G4TA	G1TB	G2TB	G3TB	G4TB	Subgroup A	Subgroup B
Cox2	Mean	1.16[a]	21.4[b]	3.95[a]	2.32[a]	1.21[a]	14.74[b]	1.03[a]	2.62[ab]	0.026	0.022
	SD	0.63	6.55	3.94	1.67	0.58	4.57	0.74	1.81		

Different superscripts in the same subgroup mean statistically significant difference between the groups.

P value ≤ 0.05 is considered statistically significant.

after exposure to chemotherapeutic drugs like MTX [40]. MTX has been reported to decrease the buffer capacity of saliva and the output of salivary IgA in patients undergoing treatment for systemic cancer [41].

In the present study, single dose of MTX injected intraperitoneally had adversely affected the internal histological structure of the rat submandibular glands after 5 days. Moreover, after 10 days of MTX injection, the salivary gland showed further loss of acinar architecture with variable sizes of nuclei, increased areas of fibrosis with inflammatory cell infiltration. This might be due to the antiproliferative and damaging effect of MTX on cells of salivary gland as it affects normal as well as abnormal cells. Results of previous studies are in coordance with these findings [21, 42, 43].

In the our study, the (MTX + Green Tea) subgroup treated for 5 days revealed marked improvement in the size of ducts with almost normal lumen size and epithelial lining. After 10 days, further improvement was noticed. This improvement may be due to the antioxidant and anti-inflammatory effect of Green Tea.

Korany and Ezzat [44] studied the prophylactic effect of Green Tea versus Black Seed on rat parotid gland after injection with fenitrothion (pesticide). The Green Tea had an ameliorating effect on the parotid gland which had relatively normal arranged structure and few cytoplasmic vacuolization after treatment with Green Tea. They explained this protective effect by antioxidant properties, scavenging of ROS, anti-inflammatory effect and its ability to fight the cytotoxic effect of the drug.

MTX led to increase in expression of COX-2 (when compared with control group) in submandibular salivary gland at 5 days with a significant statistical difference. Comparing the submandibular salivary gland in (MTX) and (MTX + Green Tea) subgroups treated for 10 days, a decrease in COX-2 expression in the latter with a significant statistical difference, indicated the anti-inflammatory effect of Green Tea and its ability to inhibit NFkB thus inhibiting the release of TNF- α , ILs and COX-2 enzyme.

Comparing the (MTX) and (MTX + Black Seed) subgroups treated for 5 and 10 days, a decrease of COX-2 expression in the treated subgroups with a significant statistically difference, indicated the anti-inflammatory effect of Black Seed.

These results agree with previous studies that showed the anti-inflammatory effect of Black Seed oil on rats and demonstrated the inhibitory effect of Black Seed on cyclooxygenase and 5-lipoxygenase pathways of arachidonate metabolism in rats [30].

Similarly, single dose of MTX injected intraperitoneally had adversely affected the internal histological structure of the rat buccal mucosa after 5 days showing epithelial hyperplasia and hyperkeratosis. Signs of inflammation were also found. After 10 days of MTX injection, hyperplasia, hyperkeratosis and inflammatory signs were profound.

This alteration of histological structure of buccal mucosa is in accordance with Ahmed et al. [45] and Mosel et al. [46] who suggested that the etiology of oral mucositis is primarily due to the damage in components of submucosal layer, which can occur before the injury of the overlying epithelium. Injury and eventual apoptosis of vascular endothelial cells result in loss of secreted epithelial growth factors such as keratinocytes growth factors, which may explain deregulation of the normal growth of mucosal epithelial cells. Similar results were seen in the examined cases of the dorsum of the tongue.

Furthermore, Rahnama et al. [47] in their study concluded that chemotherapy killed epithelial cells of mucosa causing direct damage and atrophy in the form of diffuse or localized ulcerative lesions. They explained this by the cytotoxic effect on epithelial cell layer by causing destruction of the DNA of

progenitor cells belonging to the basal cell layer. As a result, apoptosis and dysfunction of these cells occurs.

In our present study, the (MTX + Green Tea) subgroup treated for 5 days revealed a marked reduction of epithelial and keratin thickness with few, short and regular rete ridges as well as subside of inflammatory signs in the epithelium of the dorsum of the tongue. Vacuolar degeneration markedly decreased and minimal signs of inflammation were noted. After 10 days, further improvement was noted.

We found that (MTX + Black Seed) subgroup treated for 5 days revealed partial improvement in the histologic structures, showing almost normal thickness of keratinous and epithelial layers of the buccal mucosa and the dorsum of the tongue.

Immunohistochemical results revealed a positive immune reaction in all groups treated with MTX of buccal mucosa, salivary glands and dorsum of the tongue indicating the presence of severe inflammation.

Methotrexate led to increase in expression of COX-2 (when compared to the control group) in all three tissues at 5 and 10 days with a significant statistical difference.

Logan et al. [19] supported this result. They demonstrated statistically significant increased oral mucosal staining for NFkB and COX-2 following cytotoxic chemotherapy and provided further support for the role of COX-2 in the pathogenesis of mucositis by releasing matrix metalloproteinase enzymes and PGs which cause further tissue damage and exacerbate the inflammatory condition.

Comparing the (MTX), (MTX + Green Tea) and (MTX + Black Seed) subgroups treated for 5 and 10 days in the buccal mucosa, salivary glands and dorsum of tongue, a decrease of COX-2 expression in the latter treated subgroups was clear with a significant statistical difference. These results confirmed the antioxidant and anti-inflammatory effect of both Green Tea and Black Seed.

CONCLUSION

In Albino rat submandibular salivary gland, Black Seed use for 5 and 10 days and Green Tea use for 10 days had a satisfactory effect on reducing MTX induced cytotoxicity, while Green Tea use for 5 days had minor effect on MTX induced cytotoxicity.

Concerning the buccal mucosa, Green Tea use for 5 and 10 days had a counteracting effect on MTX induced cytotoxicity, while Black Seed had a minor effect.

Finally, evaluating the dorsum of the tongue, Green Tea use for 5 and 10 days and Black Seed use for 5 days also had a satisfactory effect on reducing MTX induced cytotoxicity, while Black Seed use for 10 days had a minor effect.

Recommendation

1- Further studies using of a combination of Green Tea and Black Seed are recommended.

2- Further studies using Green Tea and Black Seed as prophylaxis prior to MTX injection are recommended.

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