

Effects of Vitamin D3 on Methotrexate- Induced Jejunum Damage in Rats

Farah K. Abdul-Wahab^{*,1} and Nada N. Al-Shawi^{*}

*Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq

Abstract

Both methotrexate and vitamin D3 are used in combination for the treatment of various diseases. The aim of this study is to highlight the effect of vitamin D3 on methotrexate-induced jejunum damage using biochemical and histopathological studies. Seven groups of both sexes of rats were selected and treated as follows: (Group I and Group II) : control 1, control 2 (I.P normal saline) daily for 14 and 21 days respectively ; (Group III and Group IV) :vitamin D3 groups (500 IU/rat/day) orally for 14 and 21 days, respectively;(Group V): a single dose of methotrexate 20mg/kg, I.P injected for 4 days;(Group VI):vitamin D3 (500 IU/rat/day) single for 14 days and methotrexate (20 mg/kg I.P) injected only at day 10;(Group VII) vitamin D3 (500 IU/rat/day) orally for 21 days and methotrexate (20 mg/kg I.P) injected only at day 17; then the jejunum was removed and used for measuring malondialdehyde (MDA) content, total antioxidant capacity (TAOC) level; in addition histopathological study of jejunum tissue. Administration of vitamin D3 for 21 days and a single dose of methotrexate at day 17 resulted in non-significant difference ($P>0.05$) in MDA; while significant reduction ($P<0.05$) in the TAOC level in jejunum tissue; furthermore , sever villi damage ,crypts abscess, epithelial atrophy , mixed inflammatory cells infiltrate and goblet cells depletion were observed in comparison with methotrexate group. So the study demonstrates that vitamin D3 plays a synergistic role with methotrexate therefore the combined use of vitamin D3 and methotrexate may be used as a strategy to overcome dose limitations and side effects when use for the treatment of cancer, rheumatoid arthritis and psoriasis.

Key words: Jejunum damage, Methotrexate, Oxidative stress, Rats, Vitamin D3.

تأثير فيتامين د ٣ على تلف الصائم الناجم عن الميثوتريكسيت في الجرذان فرح قيس عبدالوهاب^{*,١} و ندى ناجي الشاوي^{*}

*فرع الادوية والسموم ، كلية الصيدلة، جامعة بغداد، بغداد، العراق.

الخلاصة.

ان كلاً من الميثوتريكسيت وفيتامين د٣ يمكن استخدامهما معا لعلاج أمراض متعددة الهدف من هذه الدراسة هو تسليط الضوء على تأثير فيتامين د٣ على تلف الصائم الناجم عن الميثوتريكسيت باستخدام الدراسات الكيميائية الحيوية والنسجية، تم اختيار سبع مجاميع من الجرذان من كلا الجنسين واعطيت على النحو التالي: (المجموعة الاولى والثانية) : مجموعة السيطرة ١ ومجموعة السيطرة ٢ زرقت داخل الصفاق (محلل الملح الطبيعي) يومياً لمدة ١٤ و ٢١ يوماً على التوالي ، (المجموعة الثالثة والرابعة): مجاميع فيتامين د٣ (٥٠٠ وحدة دولية /جرذ/يوم) فمويًا لمدة ١٤ و ٢١ يوماً على التوالي ؛ (المجموعة الخامسة): الميثوتريكسيت جرعة واحدة (٢٠ ملغم/ كغم وزن الجسم داخل الصفاق) لمدة ٤ أيام؛ (المجموعة السادسة): فيتامين د٣ (٣٠٠ وحدة دولية / جرذ/ يوم) [مرة واحدة يومياً لمدة ١٤ يوماً والميثوتريكسيت (٢٠ ملغم/كغم) الذي زرقت فقط في اليوم ١٠ داخل الصفاق؛ (المجموعة السابعة): فيتامين د٣ (٥٠٠ وحدة دولية / جرذ/ يوم) عن طريق الفم لمدة ٢١ يوماً والميثوتريكسيت (٢٠ ملغم / كغم) الذي حقن فقط في اليوم ١٧ داخل الصفاق؛ ثم تمت إزالة الصائم واستخدامه لقياس المألونداي الديهايد، والسعة الكلية المضادة للأكسدة فضلاً عن دراسة مرضية نسجية لأنسجة الصائم. نتج من إعطاء فيتامين د٣ (٣٠٠) لمدة ٢١ يوماً وجرعة واحدة من الميثوتريكسيت في اليوم ١٧ انخفاضاً غير معنوي في نسبة المألونداي الديهايد ($P>0.05$) بينما كان انخفاضاً معنوياً للسعة الكلية المضادة للأكسدة ($P<0.05$) وتلف شديد في الزغب وخراج في الاقبية وضمور الطلائية وتسلل الخلايا الالتهابية المختلطة ونضوب خلايا الكأس مقارنة بمجموعة الميثوتريكسيت. لذلك ، توضح الدراسة أن فيتامين د٣ يلعب دوراً متآزراً مع الميثوتريكسيت ، لذلك يمكن استخدامها مع فيتامين د٣ والميثوتريكسيت كاستراتيجية للتغلب على حدود الجرعة والآثار الجانبية عند علاج السرطان والتهاب المفاصل الروماتويدي والصدفية.

الكلمات المفتاحية : تلف الصائم، الميثوتريكسيت، الاكسدة ، الجرذان، فيتامين د ٣ .

¹Corresponding author E-mail: farah77kais@yahoo.com.

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Introduction

Methotrexate (MTX), a folic acid antagonist, inhibits the enzyme dihydrofolate reductase (DHFR) and it is utilized as an antineoplastic and anti-rheumatoid agent⁽¹⁾. The effects of MTX are not specific in action against tumour cells but also it can damage normal rapidly proliferating cells, namely intestinal epithelial cells⁽²⁾. The jejunal damage induced by MTX may be due to several factors including oxidative stress (OS), inflammation and apoptosis. Therefore, it is imperative to combine it with another drug, which could decrease its toxicity and increase its efficacy. It has been reported that different *in vitro* and *in vivo* studies have shown that OS and dysregulation of antioxidant enzymes can play an important role in MTX-induced jejunal damage^(3, 4). Moreover, researchers reported that there is no specific treatment for MTX-induced jejunum damage once it has occurred^(2, 5).

Vitamin D, a fat-soluble vitamin obtainable from the diet as well as produced in the skin from sunlight. Vitamin D as a precursor of a potent steroid hormone, undergoes two-step metabolism in the liver and kidney to synthesize a biologically active form, calcitriol, which binds to the vitamin D receptor (VDR)^(6, 7) that found in most cells, resulting in wide spread actions of vitamin D3 on most physiologic and pathologic processes. The primary sites of action of such vitamin are the intestine, bone, and kidneys; thus, the biologic functions of vitamin D3 are multiple and include its classic role in bone and mineral metabolism, and other non-classic actions, including cell proliferation, immunomodulation and cell differentiation⁽⁸⁾. Vitamin D3 implicated in the pathophysiology of immune-mediated diseases including multiple sclerosis (MS) and inflammatory bowel disease (IBD) and its insufficiency has been linked to higher rates of cancers including colorectal, prostate and breast cancers^(9,10).

Objective: This study aims to study the effect of vitamin D3 at two different administration durations against MTX-induced jejunal damage in rats using biochemical and histopathological studies.

Materials and Methods

Experimental animals

Forty nine (49) adult albino rats of both sexes, weighing 150-250gm were randomly allocated into seven groups (7 animals each) were used in this study. Rats were obtained from and maintained in the Animal House of the College of Pharmacy, University of Baghdad under conditions of controlled temperature. The animals were fed commercial pellets and tap water *ad libitum* throughout the experiment period. The study was approved by the Scientific- and the Ethical-

Committees of the College of Pharmacy/ University of Baghdad.

Chemicals and drugs

Methotrexate vial [(50mg/2ml vial) Milan, Italy]; Vitamin D3 [(10,000 drops), Diabase, Italy]; malondialdehyde (MDA) Elisa kit (Elabscience Biotechnology, China); total anti-oxidant capacity kit (Elabscience Biotechnology, China).

Experimental protocol

Experimental rats used in this study were randomly divided into seven groups (7 animals /group) as follows:

Group I: Control 1 [(I.P normal saline (0.9% NaCl)] daily for 14, this group served as negative control;

Group II: Control 2 (I.P 0.9% NaCl) daily for 21 days this group served as negative control;

Group III: Vitamin D3 group (500 IU/rat/day)⁽¹¹⁾ orally for 14;

Group IV: Vitamin D3 group (500 IU/rat/day)⁽¹¹⁾ orally for 21 days;

Group V: Methotrexate (MTX) single dose (20mg/kg, I.P) for 4 days, this group served as positive control⁽¹²⁾;

Group VI: Vitamin D3 orally (500 IU/rat/day) single for 14 days and methotrexate (20 mg/kg I.P), which injected only at day 10;

Group VII: Vitamin D3 (500 IU/rat/day) orally for 21 days and methotrexate (20 mg/kg I.P), which injected only at day 17.

At the end of the experiment, all rats were anesthetized by diethyl ether and sacrificed by cervical dislocation. Then, the jejunum tissue samples were taken for the determination of MDA contents, total antioxidant capacity (TAOC), and for histopathological examination.

Determination of malondialdehyde (MDA) contents

Contents of MDA in the jejunum tissue homogenate were quantitatively estimated using MDA kit based on ELISA method (#E-EL-0060; Elabscience). The content of MDA in jejunum tissue homogenate samples can be calculated by comparing the OD of the samples with the standard curve. Level of MDA is expressed as ng/mL⁽¹³⁾.

Determination of total antioxidant capacity (TAOC) level

Total antioxidant capacity (TAOC) level was measured according to the manufacturers' protocol using colorimetric method by the utilization of the total antioxidant capacity assay kit (#E-BC-K136; Elabscience). Many antioxidants in the body can reduce Fe⁺³ to Fe⁺², which in turn can form stable complex with phenanthroline substance. The TAOC level in jejunum tissue samples can be calculated by measuring the absorbance at 520 nm⁽¹⁴⁾.

Histopathological examination

The jejunum tissue samples were fixed in 10% formalin, dehydrated in graded ethanol, and

embedded in paraffin. Sections of jejunal tissue were cut with a microtome set at a thickness of 5 μm , mounted on clear glass slides, and stained with haematoxylin and eosin (H&E). Sections were examined and photographed using light microscopy (micros) and evaluated by the specialist pathologist⁽¹⁵⁾. An overall score for the severity of the jejunal damage was assessed in stained jejunum tissue sections as follows: A- villi damage (shortening and fusion); B- crypt damage; C-epithelial atrophy; D- inflammatory cells infiltrate in the lamina propria; and E- goblet cell depletion as 0, none; 1, mild; 2, moderate; 3, severe⁽¹⁶⁾.

Statistical analysis

Data were expressed as the mean \pm standard error of the mean (SEM). The statistical significance of the differences among various groups was determined by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test by SPSS statistics version 25. Differences were considered statistically significant for P -value less than 0.05.

Results

Figure 1 showed that there were non-significant differences ($P > 0.05$) in the content of MDA in jejunum tissue homogenate among Group I (control 1), Group II (control 2), Group III (vitamin D3 for 14 days) and Group IV (vitamin D3 for 21 days); while the content of MDA in the jejunum tissue homogenate in group of rats IP injected with Group V (MTX) was significantly elevated ($P < 0.05$) compared to Group I (control 1), Group II (control 2), Group III (vitamin D3 for 14 days) and Group IV (vitamin D3 for 21 days). Furthermore, although the content of MDA was reduced in Group VI (MTX+ vitamin D3 for 14 days) and Group VII (MTX+ vitamin D3 for 21 days) but still non-significantly different when each compared to MTX-treated rats (Group V) ($P > 0.05$).

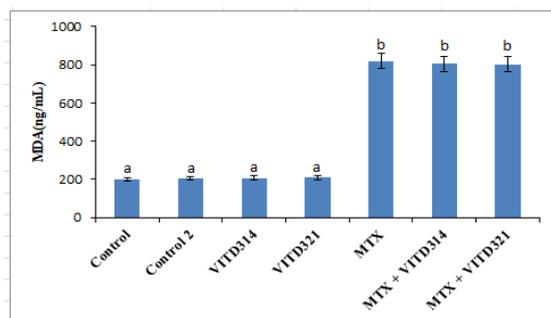


Figure 1. Effects of various treatments on malondialdehyde (MDA) contents in the jejunum tissue homogenate of rats.

Each value represents mean \pm standard error of means (SEM).

Values expressed in small letters (a, and b) are significantly different ($P < 0.05$).

Figure 2 showed that there were non-significant differences ($P > 0.05$) in the TAOC level in jejunum tissue homogenate among Group I (control 1),

Group II (control 2), Group III (vitamin D3) and Group IV (vitamin D3); furthermore, Figure 2 also showed that the level of TAOC in jejunum tissue homogenate of rats IP injected with MTX (Group V) was significantly reduced ($P < 0.05$) compared to Group I (control 1), Group II (control 2), Group III (vitamin D3) and Group IV (vitamin D3); additionally, there were significant reduction ($P < 0.05$) in the TAOC level in jejunum tissue sample in group of rats treated with MTX+VIT D3 (Group VI) and MTX+VIT D3 (Group VII) compared to MTX-treated rats (Group V) ($P < 0.05$).

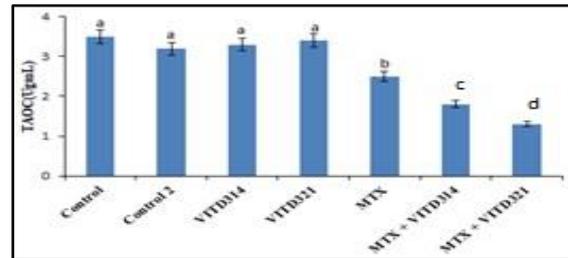


Figure 2. Effects of various treatments on total antioxidant capacity (TAOC) levels in the jejunum tissue of rats.

Each value represents mean \pm standard error of means (SEM).

Values expressed in small letters (a, b, c, and d) are significantly different ($P < 0.05$).

Histopathological examination of rats' jejunum tissue

For histopathological study in the jejunum tissue of control 1 (Group I), and control 2 (Group II) of rats, there were normal appearance of villi and crypts, with no epithelial atrophy, but a mild-moderate degree of mixed inflammatory cells infiltrate were seen within lamina propria; additionally, no goblet cell depletion was observed in Figure 3 (A and B). Furthermore, in group of rats orally-administered vitamin D3 for 14 days (Group III), there was a mild-villous and crypts damage, -epithelial atrophy, and -mixed inflammatory cells infiltrate were seen in the lamina propria; furthermore, mild goblet cells depletion was also observed (Figure 3-C). While in Group of rats orally-administered vitamin D3 for 21 days (Group IV), there are focal widening, and shorting of villi, mild crypt damage (fusion of crypt) and a mild mixed inflammatory cells infiltrate in the lamina propria and mild goblet cells depletion were observed in (Figure 3-D).

Concerning section of jejunum tissue of methotrexate-treated rats (Group V), there were moderate villous (v)- and crypts (c)- damage which was represented by shortening, widening and fusion of villi with v/c ratio 2/1; moreover, moderate epithelial atrophy, moderate inflammation is seen in lamina propria and a moderate degree of goblet cells depletion. (Figure 3-E).

In Group VI of rats-treated with methotrexate+ vitamin D3 (for 14 days), Figure 3-F showed a severe damage that represented by shorting, widening and fusion of villi (villous atrophy), and crypt damage (abscess); with villous to crypts ratio 1/1; furthermore, moderate epithelial cells atrophy, a heavy mixed inflammatory cells infiltrate, and moderate goblet cells depletion was also observed. Moreover, in Group VII of rats treated with methotrexate+ vitamin D3 (for 21 days), Figure 3-

G showed that there were severe damages that represented by loss, widening and fusion of villi with v/c ratio 1/1, severe crypt damage (the crypt epithelium is atrophied and few cells are sloughed out in the lumen of the crypt (apoptosis); additionally, severe epithelial cells atrophy, heavy mixed inflammatory cells infiltrate were seen in the lamina propria and severe goblet cells depletion.

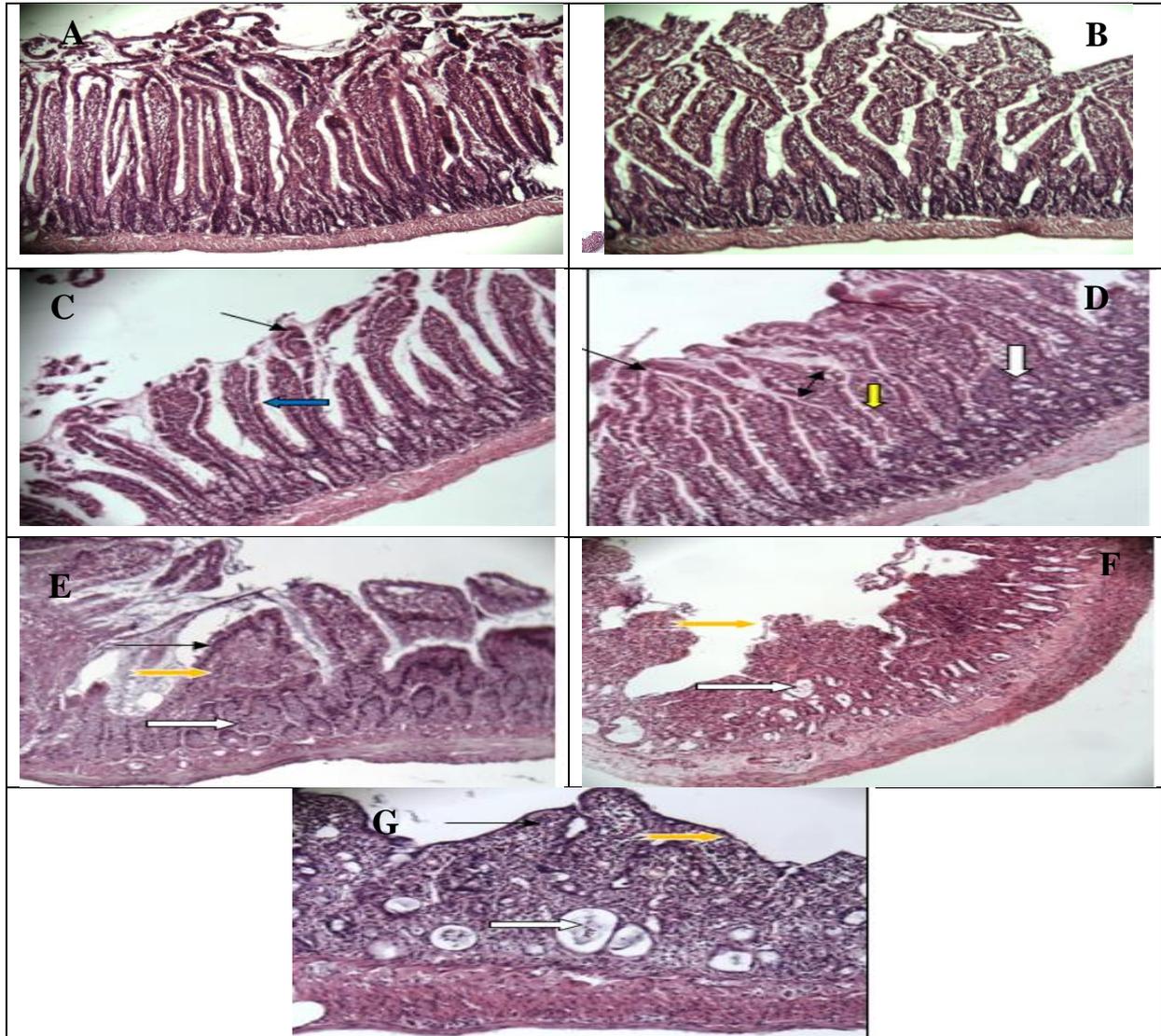


Figure 3. Sections of the jejunum tissue of various rats groups (Haematoxylin and eosin (H&E) X 10). A and B) Group I and Group II: normal appearance of villi (v) and crypts (c), no epithelial atrophy, with a mild to moderate degree of mixed inflammatory cells infiltrate is seen in lamina propria, and no goblet cell depletion. C) Group III: a mild villous, crypts damage, mild epithelial atrophy and goblet cells depletion compared to the area pointed by the large arrow which shows goblet cells. D) Group IV: focal widening () and shortening of villi, a mild crypt damage, a mild mixed inflammatory cells infiltrate in the lamina propria and the tips of villi showed mild goblet cell depletion compared to the sides of the villi where by goblet cells are still present. E) Group V: moderate villous and crypts damage represented by shortening, widening and fusion of villi with v/c ratio 2/1, moderate epithelial atrophy and moderate inflammation were seen in lamina propria and moderate goblet cells depletion. F) Group VI: severe damage that represented by shorting, widening and fusion of villi (villous atrophy) with v/c ratio 1/1, crypt abscess, moderate epithelial cells atrophy, a heavy mixed inflammatory cells infiltrate is also seen in lamina propria, and goblet cells depletion. G) Group VII: severe damage represented by loss, widening and fusion of villi with v/c ratio 1/1, crypt abscess, severe epithelial atrophy, heavy mixed inflammatory cells infiltrate is seen in the lamina propria and severe goblet cells depletion. Yellow arrows indicate villi damage, white arrows indicate crypt abscess, and blue arrow indicates goblet cells and black arrows indicate goblet cells depletion.

Discussion

The intestinal epithelium is the largest surface area of the body in contact with the external environment⁽¹⁷⁾.

Concerning MTX, such chemotherapeutic drug belongs to the antimetabolite class of medication; where, it structurally resembles folic acid, and it competitively inhibited dihydrofolate reductase (DHFR) enzyme^(18, 19). Researchers described that the therapeutic use of MTX has been limited by its impact on the rapidly-dividing cells of crypts through the inhibition of the epithelial cell proliferation and induced apoptosis in the small intestinal crypts⁽²⁰⁾; moreover, multiple factors may have role in MTX-induced small intestine damage such as the dose and the treatment duration of such drug, type of disease, in addition to apoptotic factors⁽²⁾. Additionally, several mechanisms have been hypothesized to underline small intestine injury induced by MTX such as oxidative- and nitrosative stress, up-regulation of inflammatory mediators that may promote apoptosis which in turn may augment inflammation and tissue injury of intestinal tissue which play important role in the pathogenesis of small intestine damage induce by MTX in rat^(21, 22).

Regarding the oxidative stress marker malondialdehyde (MDA), which is an end product of lipid peroxidation an events in the cells are recognized as an indicator of oxidative stress⁽²³⁾.

Figure 1 showed that there were non-significant differences ($P>0.05$) in the content of MDA in jejunum tissue homogenate among Group I (control 1), Group II (control 2), Group III (vitamin D3) and Group IV (vitamin D3); while, in group of rats IP injected with MTX 20mg/kg (Group V) caused elevation in MDA contents accompanied by the reduction of TAOC level in rats' jejunum tissue homogenate compared to control1 (Group I), control 2 (Group II), vitamin D3-orally administered rats for 14 days (Group III) and vitamin D3-orally administered rats for 21 (Group IV), this comes in line with previous animal and clinical studies, which demonstrated that OS and lipid peroxidation are hallmarks of MTX-induced jejunum damage⁽²⁴⁻²⁶⁾; furthermore, authors reported that the cell damage can be initiated by the reaction of free radicals with biological macromolecules producing lipid peroxides with the depletion of first-line antioxidant enzyme systems, including reduced glutathione (GSH)^(3, 27). Although there were reduction in MDA contents in rats' jejunum homogenate that treated with MTX with vitamin D3 orally-administered for 14 days (Group VI) and MTX with vitamin D3 orally-administered for 21 days (group VII) but still non-significantly different when each compared to MTX-treated rats (Group V). On the other hand, results of this study showed that the TAOC level

was significantly reduced in groups VI and VII rats each compared to Groups V rats as shown in figure 2.

For histopathological study in the jejunum tissue of control 1 (Group I), and control 2 (Group II) of rat treated with normal saline for 14 and 21 days, respectively, there were normal appearance of villi and crypts, with no epithelial atrophy, but a mild-moderate degree of mixed inflammatory cells infiltrate were seen within lamina propria; additionally, no goblet cell depletion was observed in Figure 3 (A and B). Furthermore, in group of rats orally-administered vitamin D3 for 14 days (Group III), there was a mild -villous and crypts damage, -epithelial atrophy, and -mixed inflammatory cells infiltrate were seen in the lamina propria; furthermore, goblet cells depletion was also observed [Figure 3-C]. While in Group of rats orally-administered vitamin D3 for 21 days (Group IV), there are focal widening, and shorting of villi, mild crypt damage (fusion of crypt) and a mild mixed inflammatory cells infiltrate in the lamina propria were observed in (Figure 3-D). The suggestion concerning the effect of vitamin D3 may be due to its pro-oxidant effect that caused an increase in ROS, which in turn may decrease GSH level; thus OS can consequently be resulted in cells.

In addition, this study showed that there were histopathological changes in the jejunum tissue of rat treated with MTX for 4 days which characterized by moderate villous damage that represented by shortening, widening and fusion of villi and crypts damage with v/c ratio 2/1, moderate epithelial atrophy and moderate inflammation were also seen in the lamina propria; furthermore, a moderate degree of goblet cells depletion is seen compared to Group I, Group II, Group III, and Group IV rats; and these changes are consistent with that observed in previous studies^(11, 28). Authors mentioned that MTX act as a pro-oxidant that may cause depletion of the tetrahydrofolate, suppressed DNA synthesis, inhibited epithelial proliferation, and induced apoptosis in the small intestinal crypt⁽⁴⁾. In addition, the release of free radicals by moderate infiltrate inflammatory cells in lamina propria may have a role in jejunum pathogenesis and this comes in line with previous study⁽²⁹⁾.

Moreover, many authors described that the oxidative damage induced by MTX in jejunum tissue can be prevented by different vitamins and natural plant extracts that may act as cytoprotectors to protect normal cells of the jejunum from MTX damage^(2, 5); but, results of the current study showed that histopathological jejunum sections of rats administered vitamin D3 for 14 days and MTX at day 10 (Group VI) (Figure 3-F); where severe damage that represented by loss, widening and fusion of villi, crypt abscess, severe epithelial cells atrophy, a heavy mixed inflammatory cells infiltrate were seen in the lamina propria and severe goblet cells depletion (Figure 3-G). Flanagan L, *et al* (2016) reported that vitamin D3 can synergistically act with MTX; where, vitamin D3 can induce apoptosis which may be due to the action of such vitamin as pro-oxidant and caused an induction of reactive oxygen species (ROS) by altering redox state of cell⁽³⁰⁾. In addition, Abu el Maaty MA and Wöfl S (2017) described that vitamin D3 can enhance the activity of MTX by increasing intracellular availability⁽³¹⁾. Additionally, Zhao R *et al* in 2013 demonstrated that the intestinal absorption of MTX and folate is mostly mediated by the proton-coupled folate transporter (PCFT), which mainly expressed in the proximal part of the small intestine at the apical brush-border of enterocytes' membrane; and vitamin D3 can increase expression of intestinal PCFT and an enhancement of cellular folate uptake⁽³²⁾. Moreover, the potential mechanism for increasing intestinal absorption of MTX can be brought about via simultaneous treatment of MTX with vitamin D3; in other word, vitamin D3 can affect the bioavailability of MTX⁽³³⁾; additionally, vitamin D3-MTX interaction was reported to be through transporters; where, such vitamin can alter the pharmacokinetics and pharmacodynamics of MTX that can result in variability of efficacy and involved in the MTX disposition in the body and in the regulation of intracellular metabolism in target cells^(31, 34). Thus, beneficial effects may be brought about when vitamin D3 and MTX used together for the treatment of different diseases such as cancer, inflammatory bowel disease, rheumatoid arthritis (RA), and psoriasis by decreasing the dose of MTX so decrease side effects and increase efficiency; but, adverse effects instead of beneficial effects were observed in this study.

Conclusion

The present study demonstrates that vitamin D3 plays a synergistic role with methotrexate by causing histopathological damage in jejunum tissue of rats; where, vitamin D3 can act as pro-oxidant and/or it can enhance the entrance of MTX inside the cell; and, adverse effects resulted from combination of vitamin D3 and MTX are observed in this study.

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