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Ameliorative Effect of Linagliptin Alone and in Combination with Methotrexate against Formaldehyde-Induced Arthritis in Rats: Biochemical and Histopathological Study

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ABSTRACT

Background: The hallmark of rheumatoid arthritis (RA), is the inflammatory cells infiltration into hyperplastic synovial tissue that leads to the eventual degeneration of bone and articular cartilage. The first traditional disease-modifying anti-rheumatic medication, methotrexate (MTX), is linked to immune responses and may reduce articular damage in RA. Effective half- of therapeutic dose of MTX is essential due to its numerous undesirable side effects.

Aim and methods: The anti-arthritic effects of linagliptin (LIN) at 5 mg/kg/day oral starting at day 4 after injection with formaldehyde (2% v/v) into the subplanter surface of a left hind paw at 1 and 3 days of the study for four weeks was evaluated alone, and in combination with a half-dose of MTX (1 mg per kg body weight IP once weekly). Serum level of rheumatoid factor (RF), anti-cyclic citrullinated peptide (anti-CCP), C-reactive protein (CRP), oxidative stress mediators, and serum apoptotic markers were assessed. Moreover, hind paw histopathology and cyclooxygenase (COX) expression by IHC were examined in this study.

Results: Rats treated with both half -dose of MTX and LIN showed better results as full dose MTX arthritic-treated rats, and is superior if compared to LIN alone. Combined therapy exhibited improvement of all above mentioned parameters. Furthermore, the rats received dual therapy demonstrated improvement of histopathological alterations. **Conclusion:** Combination of LIN and half -dose of MTX has synergetic effect so, it is beneficial in the management of RA as the standard group (full dose of MTX).

Keywords: Methotrexate, Linagliptin, Arthritis, COX.

INTRODUCTION

Rheumatoid arthritis (RA) is chronic inflammatory autoimmune disease can affect every joint in the body, but it is most common in the tiny joints of the extremities. In developed nations, 0.5–1.0% of individuals suffer from RA, with about 5–50 of new cases per 100,000 people each year [(1)]. Joint stiffness and edema are the most typical signs of RA. The hallmark of RA is continuous arthrosynovitis, which results in bone degeneration, cartilage degradation, and joint deformity. These conditions ultimately cause disability, a decline in quality of life, and early mortality. As a result, it is crucial to create an efficient therapeutic approach that mange the disease, its consequences, and related conditions [(2)].

The inflammatory synovium of RA contains a significant T - lymphocytes. In order to produce a complex network of different secreted cytokines, including IL-18, IL-1β, TNF-α, IL-15, and IL-17, inflammatory cells, and fibroblasts. The most accurate indicators of RA diseases are anti-cyclic citrullinated peptide (anti-CCP) as well as rheumatoid factor (RF) [(3)].

According to **Ekinci-Akdemi *et al.*** [(4)], methotrexate (MTX), a traditional folate antimetabolite, is frequently prescribed in clinical settings at lower dosages to treat inflammatory and immunological conditions such as psoriasis and ectopic pregnancy. It is used to treat RA because of its capacity to selectively down-regulate B-cell and T-cell activation and prevent IL-B from binding to cell surface receptors [(5)].

Long-term use of MTX has been linked to a number of serious organ toxicities, including those in the kidney, testis, liver and central nervous system, which restricts its clinical uses [(6)]. MTX toxicity can be explained by a number of mechanisms, such as the deregulation of antioxidant defense and the nuclear factor kappa B stimulation, which up regulates certain genes that produce pro-inflammatory mediators [(7)]. One of most potent dipeptidyl peptidase-IV (DPP-IV) inhibitors is linagliptin (LIN). It is used to treat type 2 diabetes mellitus (T2DM). In addition to its ability to lower blood sugar, LIN has shown notable anti-inflammatory properties in preclinical studies of neurodegenerative diseases, cardiomyopathy, and nephropathy [(8)]. It has been demonstrated that DPP-IV enzymes play a significant role in the anti-inflammatory immune response by breaking down pro inflammatory molecules like TNF-α, IL-1, and IL-2 [(1)]. The goal of this study was to ascertain whether LIN, either monotherapy or in combination with half of therapeutic dose of MTX, had anti-arthritic effects against formaldehyde-induced RA rats model.

MATERIAL AND METHODS

2.1. Drugs and Chemicals: Sigma-Aldrich Chemical Co., St. Louis, MO, USA, supplied methotrexate, Boehringer

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Ingelheim (Ridgefield, CT, USA) supplied linagliptin (Trajenta) that was liquefied in carboxymethyl cellulose (CMC) 1% and neutral 10% formalin solution (El Gomhorya Pharmaceutical Chemical Co., Egypt, provided.

2.2. Animals: Male rats aged 12 weeks, weighing 200 ± 20 g were used in this study. They were gotten from an experimental animal breeding farm (Helwan, Cairo). They were kept in the animal house at department of Pharmacology, Benha Faculty of Medicine. Every six animal were housed in a cage, in a well-ventilated area at room temperature. To guarantee complete acclimatization and prevent administration stress, they were manually handled for 30 minutes for seven days, and they were given free water and standard food. The animals were cared for throughout the experiment in accordance with Benha Faculty of Medicine's experimental animal handling protocol.

Experimental design: Six groups of six animals each were randomly selected from the thirty-six rats. Rats in group I (control) received CMC 1% as vehicle. Group II (linagliptin group (LIN)) was given LIN dissolved in CMC orally at a dose of 5 mg/kg/day [9] for continuous four weeks. Group III (arthritic non-treated) rats that received 0.1 ml injection of formaldehyde into the subplanter surface of a left hind paw at the first and third days of the study [10, 11]**.** Group IV (MTX-treated arthritis) (standard); rats received an intraperitoneal (I.P.) injection of MTX (2 mg per kg weekly) [12] at the fourth day of the study after injection with formaldehyde for four weeks. Rats in group V (LIN-treated arthritis) were given LIN by oral gavage on the fourth day of the study following a formaldehyde injection for four weeks at a dose of 5 mg/kg/day [9]**.** Group VI (LIN + ½ dose of MTX-treated arthritis) rats, which received a combination of LIN at the previously mentioned dose and a ½ dose of MTX (1 mg/kg, once weekly IP) at the fourth day of the study after formaldehyde injection for same period.

Ethical approval: All aspects of this study were approved by the Ethical Committee of Faculty of Medicine, Benha University (Under number RC 15-12-2024).

3.1 Evaluation of arthritis

- *Arthritic score:* Five-grade system scoring was used to measure the arthritis score in order to determine the severity of the condition. 0 : no affection, 1: redness and slight swelling limited to ankle joints, 2: mild swelling and redness spreading from the ankle joint to mid-foot (tarsal), 3: moderate swelling spreading from the ankle joint to metatarsal joint with redness and 4: redness associated with severe swelling involving the ankle, foot, and digits. The average scores for arthritis were Calculated [13]**.**

- *Paw swelling:* at the end of the study, a digital Vernier caliper was used to measure the paw thickness on both side of each rat in the study [14]**.**

3.2 Samples collection: The animals were euthanized at the termination of this study. Centrifugation was done at 3000 rpm for 10 minutes after extraction of blood via cardiac puncture. Serum was then stored at 20 °C room temperature for subsequent biochemical analysis. Animals hind paws were taken out and preserved in 10% formalin for histopathological examination.

3.3 Serum inflammatory markers assay: The level of rheumatic factor (RF) was measured using the ELISA technique [15]. The C-reactive protein (CRP) level was determined using the method of **Andersen and McCarthy [16]**, as outlined in the TULIP DIAGNOSTICS kit for C-reactive protein. Serum concentrations of TNF-α as well as IL-1β were assessed using an ELISA kit (Minneapolis, MN, USA)**.** Serum samples were examined for antibodies against cyclic citrullinated peptide (anti-CCP) with ELISA technique.

3.4 Serum oxidative stress markers assay: Reduced glutathione (GSH) in addition to malondialdehyde (MDA) serum concentrations were measured using ELISA kits obtained from Life Span Biosciences Company (LS.Bio) in North America, with the following catalog numbers: MDA (ab255730) and MyBiosource for GSH (MBS265966), following the manufacturer's instructions.

3.5 Apoptotic &antiapoptotic markers measurement: The quantitative calculation of caspase-3 level was accomplished via a caspase-3 colorimetric assay kits (Catalog no. 907-013) and BCL-2 ELISA Kit (CSB-E08854r) for BCL-2 in serum. A kit from MyBioSource and CUSABIO TECHNOLOGY were used to assess serum caspase-3 and BCL-2 levels respectively according to the manufacturer's instructions.

3.6 Histopathology and immunohistochemical (IHC) analysis: Hind paws were excised from the animals, the coved muscle was precisely detached, and the tissues were preserved overnight at 4 °C in 10% formalin. Following three washes with ice-cold PBS, the samples underwent decalcification at 4 °C using EDTA 10% (pH 7.4) for 3weeks before being embedded in paraffin [17]**.** Using a microtome, these blocks were sliced to produce sectors with 10 µm thickness. Tissues were stained with hematoxylin and eosin, and final slides were examined for pannus development, swelling occurrence, and bone attrition [18] **.** For IHC, slices were further stained with individual primary antibodies against COX-2 (ab15191, 1:100, Abcam) overnight at 4 °C, and then sections were counterstained with hematoxylin. The total number of positively stained cells was calculated in four randomly selected sectors of the subchondral bone in five successive slices from each animal in every group [17]**.**

Statistical analysis

The data of our work were tabulated and analyzed using SPSS version 25 software (SPSS Inc., Chicago, ILL Company). The data were represented using standard deviations (SD) and mean for each biomarker that was analyzed. Data were examined for differences using the one-way analysis of variance test (ANOVA). A significant ANOVA test and post hoc multiple comparisons using Bonferroni testing were used to identify significant pairings. In this study, $P \leq 0.05$ was estimated significant.

RESULTS

Ameliorative effect of LIN on gross appearance, paw swelling and arthritic score: Induction of rheumatoid arthritis in rats by formaldehyde resulted in significant increase arthritic score and paw thickness compared to that in control groups. These parameters were significantly improved in all treated groups compared to arthritic rats. However, rats received combined therapy showed much better results (Figur1 & figure 2 A, B).

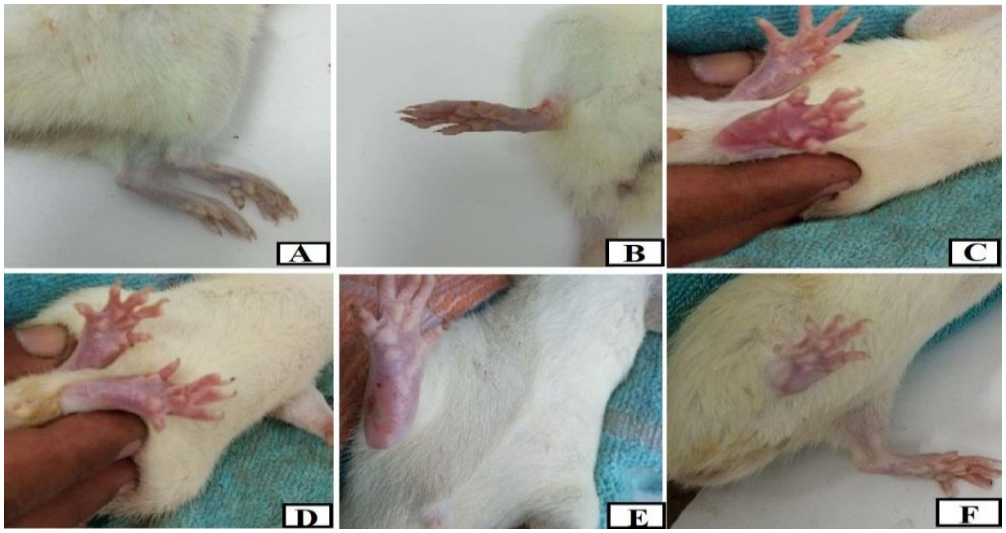
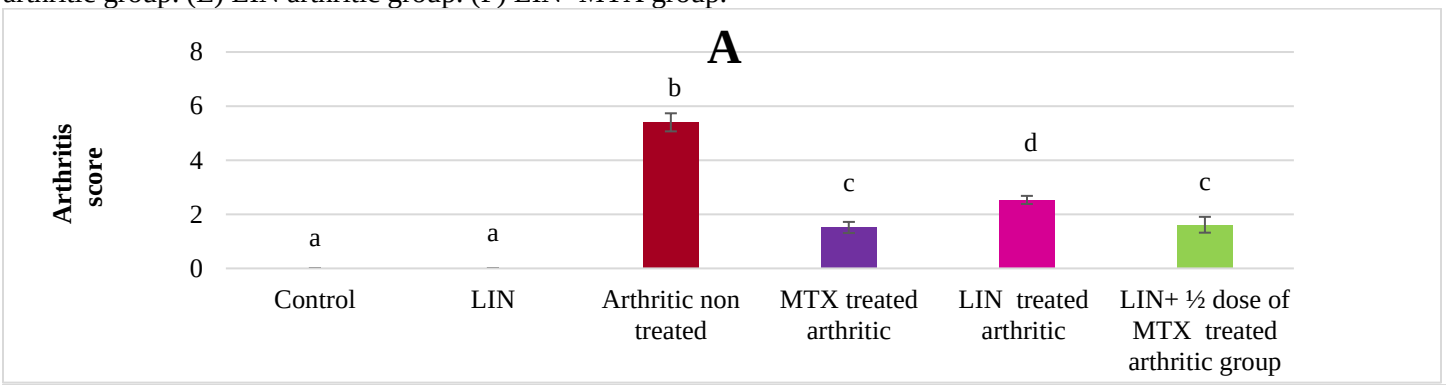


Figure (1): Gross assessment of rat's hind paws: (A) Control group. (B) LIN group. (C) Arthritic group. (D) MTX arthritic group. (E) LIN arthritic group. (F) LIN+MTX group.



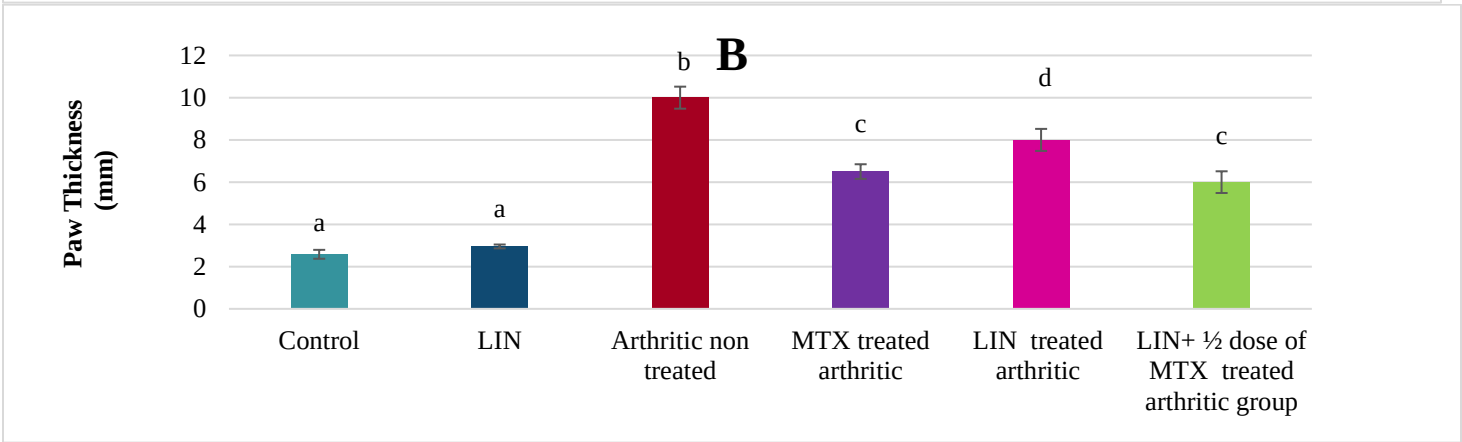


Figure (2): Ameliorative effect of LIN alone and in combination with MTX in formaldehyde -induced arthritis: (A) Arthritis score, (B) Paw thickness. Values are stated as means ± SD (n = 6). ANOVA test was followed by post hoc multiple comparisons test. Values with dissimilar letters are important at $P < 0.05$.

Ameliorative effect of LIN on serum rheumatoid arthritis markers: Rats of rheumatoid arthritis group displayed substantial increase in RF, CRP and anti-CCP compared to that in control groups. These parameters were considerably decreased in all treated groups compared to arthritic group. There was non-significant variance between MTX-treated group and LIN+ MTX combination group (P >0.05) (Table 1).

Table (1): Ameliorative effect of LIN on serum rheumatoid arthritis markers changes

	Control	LIN	Arthritic non treated	MTX treated arthritic	LIN treated arthritic	LIN+ ½ dose of MTX treated arthritic group
RF(IU\ml)	2.73^{a} ±0 .31	2.56^{a} ± 0.38	13.05^{b} ± 1.18	5.0^{c} ± 3.7	7.54^{d} ± 1.25	5.54^{c} ± 1.25
CRP(ng\ml)	2.65^{a} ± 0.378	2.50^{a} ± 0.42	15.67^{b} ± 2.86	6.12^{c} ± **0**.88	9.42^{d} ± 0.94	6.42^{c} ±0 .95
Anti-CCP (U\ml)	5.15^{a} ± 0.695	5.05^{a} ±0 .67	40.17^{b} ± 2.86	18.75^{c} ± 0.95	25.70^{d} ± 1.08	16.08^{c} ± 3.63

Values presented as means ± SD for six different animals for each group. Values with dissimilar letters are statistically variant at P < 0.05. SD, standard deviation.

Ameliorative effect of LIN on serum oxidative stress indicators: Diseased rats exhibited momentous upsurge (p<0.05) in MDA level and substantial reduction (p<0.05) in GSH level, compared to control group. However, serum concentrations of these markers were significantly improved (p<0.05) in all treated groups if compared to diseased group. There was no significant variance (p<0.05) in the concentrations of previous mentioned parameters between MTX-treated group and combination group. The combined treatment group displayed significant improvement (p<0.05) in the levels of these markers when compared to the arthritis-only rats (Table 2).

Ameliorative effect of LIN on serum apoptosis markers changes: The serum level of caspase- 3 was considerably augmented (p<0.05) in arthritic group, whereas Bcl-2 was considerably reduced (p<0.05) compared to the levels observed for the control group. Treated groups showed marked decrease (p<0.05) of caspase-3 serum level and substantially elevated (p<0.05) serum level of Bcl-2, compared to arthritic group. Caspase-3 and Bcl-2 levels showed no significant variance (p>0.05) between MTX-treated group and combination group. The combination group resulted in higher momentous difference (p<0.05) than linagliptin alone treated group (Table 2).

Table (2): Ameliorative effect of LIN on serum oxidative markers and apoptotic –anti apoptotic markers changes

	Control	LIN	Arthritic non treated	MTX treated arthritic	LIN treated arthritic	LIN+ ½ dose of MTX treated arthritic group
MDA((ng/mL)	7.24^{a}± 1.32	6.84^{a} ± 1.20	25.70^{b} ± 1.12	12.5^{c}± 1.17	18.95^{d} ± 1.01	11.55^{c} ± 1.16
Serum GSH(µg\ml)	25.70^{a}± 1.08	24.70^{a}±1.15	5.54^{b} ± 1.32	18.45^{c} ±0 .95	13.05^{d} ± 1.18	17.32^{c} ±1.02
Serum Bcl2(ng\ml)	11.15^{a} ± 0.77	11.55^{a} ± 0.73	2.10^{b} ± 0.26	7.33^{c} ± 0.59	5.10^{d} ± 0.58	7.83^{c} ±0 .59
Caspase-3(pg\ml)	1.14^{a}±0 .12	1.54^{a} ±0 .24	13.95^{b} ± 1.84	4.83^{c}±0 .61	9.33^{d} ± 0.59	5.28^{c} ± 0.57

Values presented as means ± SD for six different animals for each group. Values with dissimilar letters are statistically variant at P < 0.05. SD, standard deviation.

Ameliorative effect of LIN on serum inflammatory and anti-inflammatory markers change:

The concentrations of TNF-α and lL-1B were considerably elevated ($p<0.05$) in untreated arthritic group compared to control group. There was a significant reduction ($p<0.05$) in the levels of the previous mentioned parameters in all treated groups relative to untreated arthritic group. There was no statistical difference ($p > 0.05$) between MTX treated group and combination group. The combination group exhibited better significant effect ($p<0.05$) more than linagliptin-treated group (Fig 3 A, B). Regarding the level of IL-10, it was found that it was significant reduced ($p<0.05$) in untreated arthritic group in relation to the control group. All treated groups resulted in momentous rise ($p<0.05$) in serum IL-10, if compared to untreated arthritic group.

In contrast, there was no significant alteration ($p>0.05$) between MTX-treated group and combination group. The serum IL-10 level in combination group was observed to be considerably greater ($p<0.05$) than linagliptin-treated group, suggesting a better antioxidant activity (Fig 3 C).

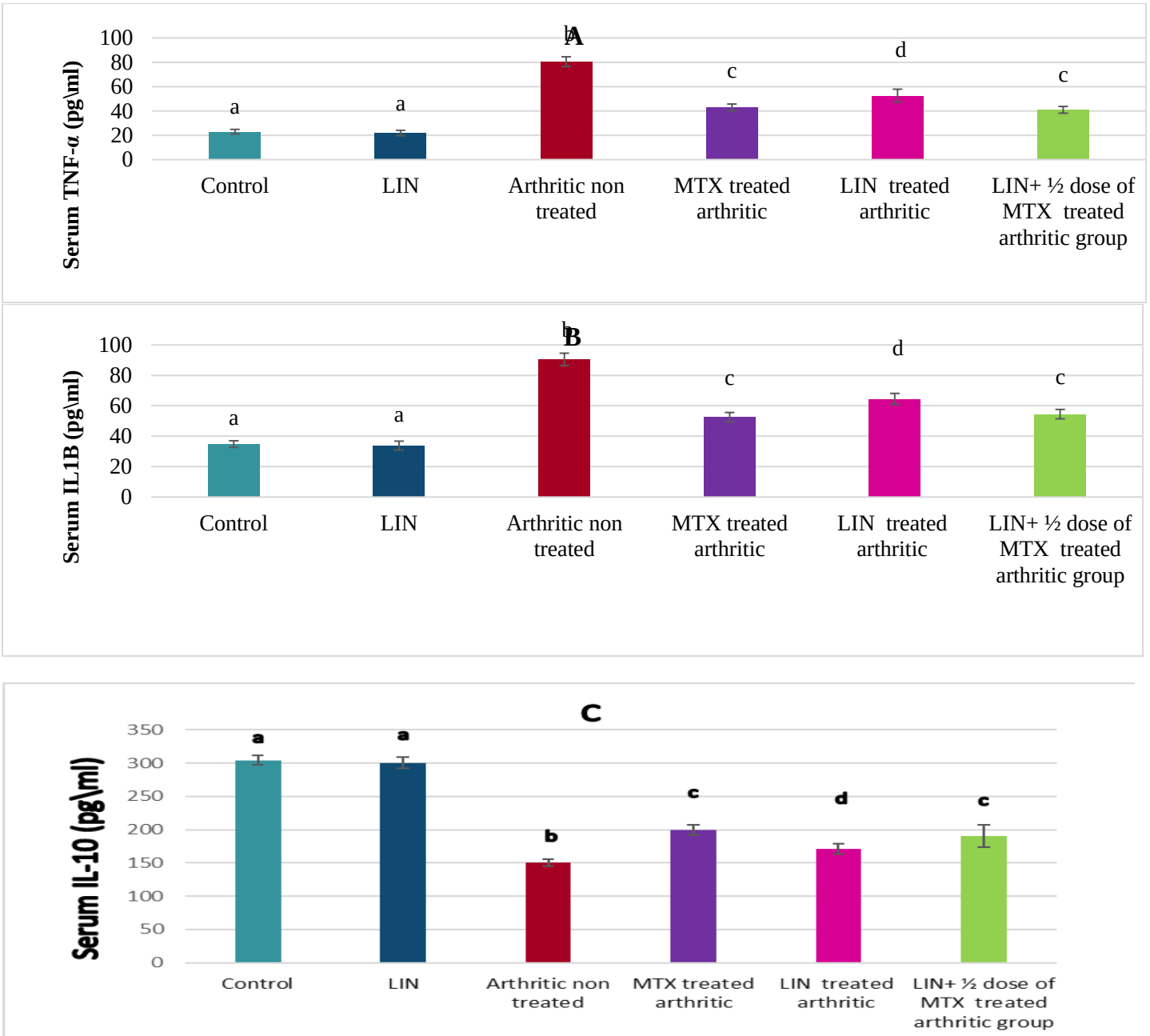


Figure (3): Ameliorative effect of LIN on serum inflammatory and anti- inflammatory markers changes. (A) Serum TNF-α, (B) serum IL-1 and (C) serum IL-10. Values are stated as means ± SD (n = 6). ANOVA test was followed by post hoc multiple comparisons test. Values with dissimilar letters are important at $P < 0.05$.

HISTOLOGICAL RESULTS

H & E staining: H & E stained sections from the control rats showed normal joint histological architecture with characteristic bone structure containing osteocytes in their lacunae. Flattened synovial membrane and normal bone marrow were also observed (Figure 4A). Section from LIN group displayed normal bone structure of the joint containing osteocytes in their lacunae and normal structure of Haversian system. Flattened synovial membrane and normal small sized blood vessels also seen (Figure 4B).

Sections from arthritic animals presented disturbed joint structure with bone erosion and multiple empty spaces. There were congested blood vessels, sever cellular infiltration, and sever fibrosis (Figure 4C).

Sections from MTX arthritic rats showed obvious improvement in the joint construction with normal cartilage structure having chondrocytes sited in their lacunae. Flattened synovial membrane more or less like control group, but still there are cellular infiltration (Figure 4D). Sections from LIN arthritic rats revealed relatively normal bone and cartilage structure of the joint. However, there was slight separation at synovial membrane, dilated blood vessels, and cellular infiltration (Figure 4E).

Sections from LIN+ MTX-treated rats displayed normal joint structure similar to that of the control animals. Typical bone structure containing osteocytes in their lacunae and numerous blood sinusoids were seen. Nevertheless, there is still less cellular infiltration (Figure 4F).

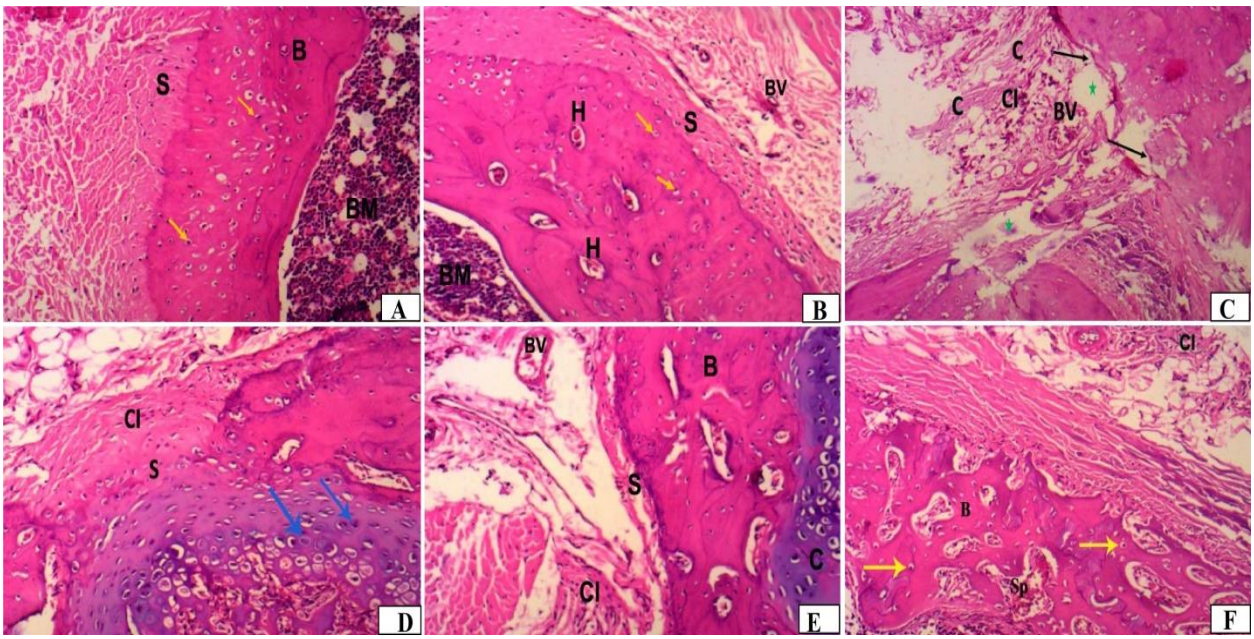


Figure (4): Photomicrographs of a cut sections in the joint of the hind paw of: (A) Control rats showing normal bone structure (B) Containing osteocytes in their lacunae (yellow arrow), flattened synovial membrane (S) and normal bone marrow (BM). (B) LIN group showing normal bone structure containing osteocytes in their lacunae (yellow arrow), normal structure of Haversian system (H), flattened synovial membrane (S) and normal bone marrow (BM). Notice, normal small sized blood vessels were also seen (BV). (C) Arthritic animals displaying disturbed joint structure with bone erosion (black arrow), multiple empty spaces (Star), congested blood vessels (BV) and sever cellular infiltration (CI). Notice, sever fibrosis (C) also seen (collagen fibers). (D) MTX arthritic rats displaying normal cartilage structure with chondrocytes sited in their lacunae (Blue arrow), flattened synovial membrane (S) more or less like control group, but still there are cellular infiltration (CI). (E) LIN arthritic rats demonstrated relatively normal bone (B) and cartilage (C) structure. There was slight separation at synovial membrane (S), dilated blood vessels (BV) and cellular infiltration (CI). (F) LIN+ MTX rats demonstrated normal structure like the control animals; typical bone structure (B) containing osteocytes in their lacunae (yellow arrow) and numerous blood sinusoidal spaces (Sp) . However, there is still less cellular infiltration (CI) (**H & E 200).**

Immunohistochemical staining: Immunostained sections from the control group and LIN group displayed negative COX-2 immune expression in the osteocytes. On the other hand, arthritic group presented intense COX-2 immunoreactivity in the majority of the osteocytes. There were reduced COX-2 immunoreactivity in MTX arthritic group as well as in LIN arthritic group. While, combination treatment of LIN+MTX in group VI displayed weak COX-2 immune expression in few osteocytes (Figures 5A-F).

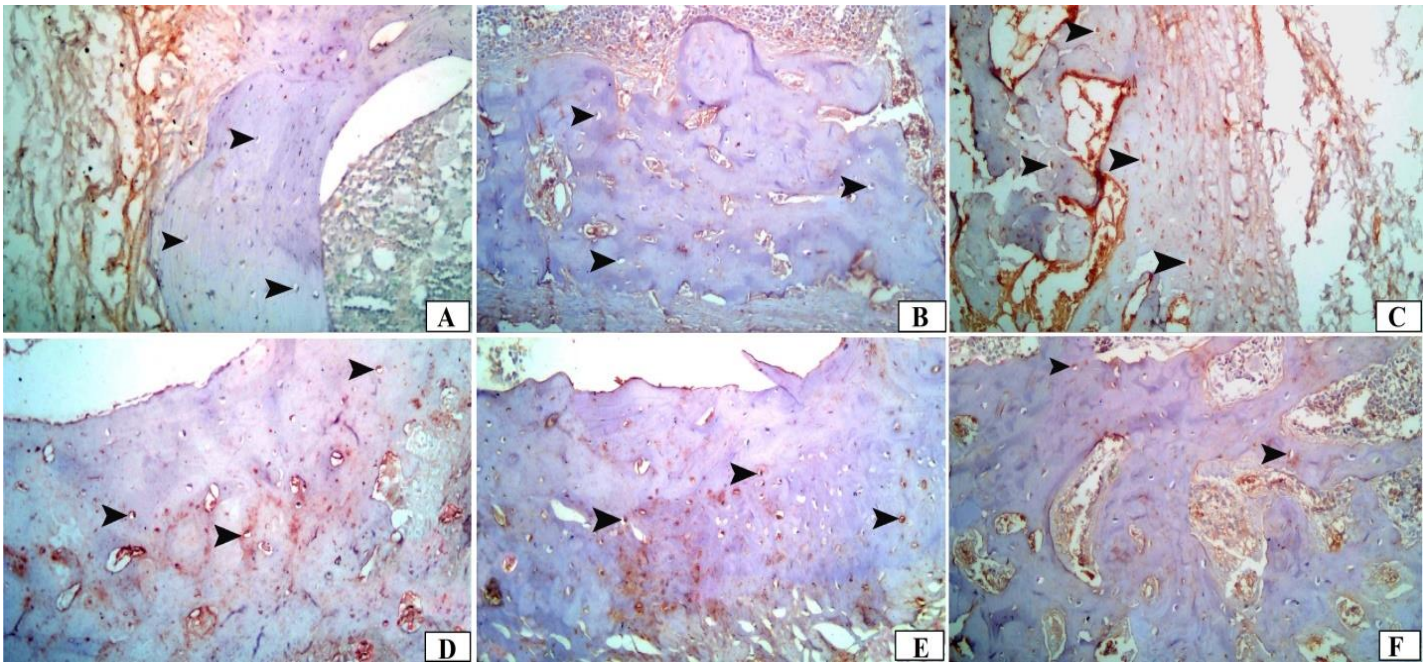


Figure (5): Photomicrographs of rat's hind paw joint immunohistochemistry for COX-2 immune expression (x200): (A) Control rat joint displayed negative COX-2 immunoreactivity in the osteocytes (arrowhead). (B) LIN group presented negative COX-2 immunoreactivity in the osteocytes (arrowhead). (C) Arthritic group presented intense COX-2 immunoreactivity in the majority of the osteocytes (arrowhead). (D) MTX arthritic group showed reduced COX-2 immunoreactivity in the osteocytes (arrowhead). (E) LIN arthritic group showed moderate expression of COX-2 in some osteocytes (arrowhead). While, (LIN+MTX) group (F) displayed weak COX-2 immune expression in few osteocytes (arrow head).

Morphometric result: Regarding the average percentage of COX 2 immune positive cells, the arthritic non-treated group revealed considerably greater value ($P \leq 0.05$) than the control groups. On the other hand, all treated groups exhibited momentous decline in this percentage in relation to the arthritic group. Interestingly, the combination of LIN and ½ dose of MTX in group VI showed the best results (Figure 6)

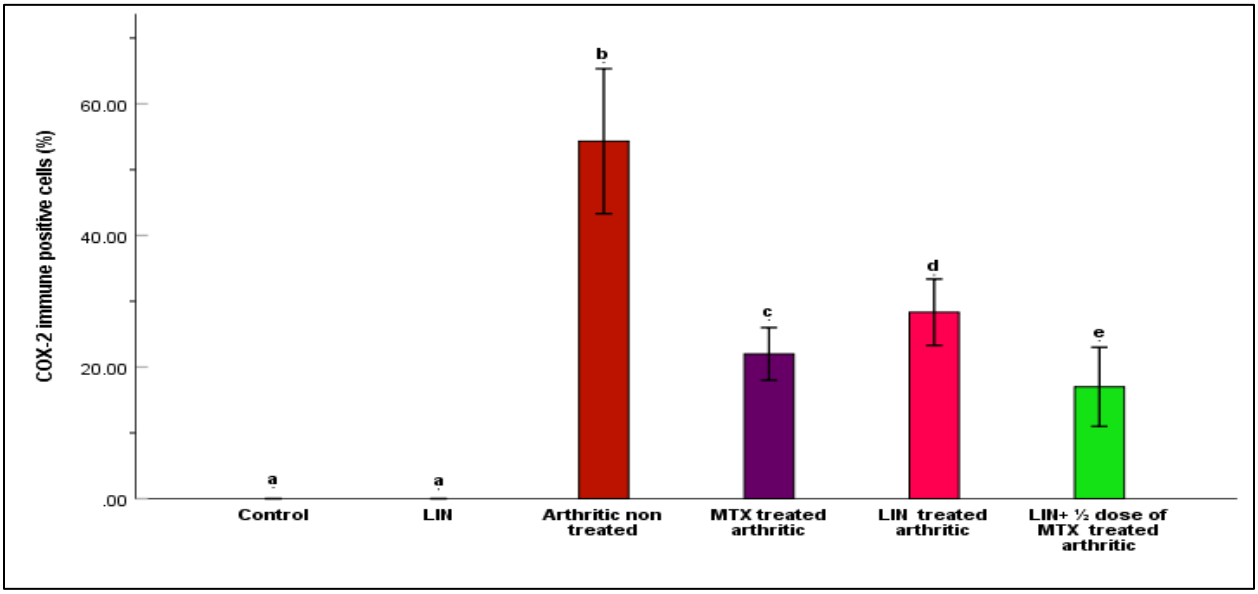


Figure (6): Presenting the average percentage of COX 2 immune positive cells in different experimental groups. ANOVA test was followed by post hoc multiple comparisons test. At $P < 0.05$, values with dissimilar letters are important.

DISCUSSION

Rheumatoid arthritis, the systemic autoimmune illness, is recognized by inflammatory arthritis in addition to involvement of extra-articular tissues [19]. The key component of rheumatoid arthritis therapy is methotrexate (MTX). However, long-term MTX usage has been linked to a number of significant organ toxicity [20]. Thus, new treatment approaches are desperately required to lower the dosage and, therefore, the adverse effects of MTX.

The present study was aimed to evaluate the anti-arthritic activity of LIN alone and in combined with half of therapeutic dose of MTX against formaldehyde-induced arthritis in rats.

In this investigation, rats exposed to formaldehyde exhibited substantial increase in arthritic score, paw thickness, RF, CRP and anti-CCP compared to that in control groups. These outcomes are in agreement with **Bose *et al.*** [21] who illustrated the relationship between the paw edema and cellular infiltration, increase in fluid outflow, as well as vascular permeability in the afflicted region. Additionally, **Kumar *et al.*** [11] reported that assessing paw swelling is the simplest method to estimate the severity of the disease and provides a general metric for evaluating the effectiveness of anti-arthritic treatments. Our results are also in line with **Farrukh *et al.*** [13] who found that administration of formaldehyde resulted in an unusual rise in the concentrations of C reactive protein and rheumatoid factor, along with an upsurge in the arthritic index in the affected animals. According to other author, formaldehyde triggers arthritis by altering proteins at the injection site, leading to an immune response against the altered substances [10].

On the other hand, our work demonstrated that administration of MTX and LIN alone or in combination with ½ dose of MTX, caused significant reduction of all above parameters but the combined therapy caused better results. This is covenant with **Mansour *et al.*** [22] who documented that MTX considerably suppressed the swelling in the rats hind paw and improved all the established parameters of rheumatoid arthritis as well as arthritic score. The DPP-4 inhibitors probable mechanisms comprise the pro inflammatory cytokines suppression, macrophage regulation and the arrangement of the function and differentiation of T-cells [23].

Either a decrease in the body's natural antioxidant defense system or an increase in reactive oxygen species (ROS) production leads to oxidative stress. Because ROS and reactive nitrogen species (RNS) cause membrane oxidation, irreversible protein and DNA damage, cartilage degradation, and bone resorption, they potentially play a role in the pathophysiology of RA [24]. In the current study, induction of RA by formaldehyde in rats model produced significant rise of serum level of MDA with significant decrease in serum GSH level. These findings are supported by **Sharma *et al.*** [25] who mentioned that rats with formaldehyde-induced arthritis showed a substantial rise in MDA levels, which was caused by lipid peroxidation and enhanced ROS formation.

While administration of MTX and LIN alone or in combination with MTX caused improvement of oxidative stress markers and the best results were with combination therapy. This goes with the study by **Arab *et al.*** [23] who revealed that LIN reduced oxidative stress in the colonic mucosa of inflammatory bowel disease rat model. Moreover, it has been observed that LIN diminished the oxidative stress and suppressed ROS via activating the signaling pathway Nrf2/HO-1 in an acute kidney damage rat mode [26].

The pathogenesis of rheumatoid arthritis is significantly influenced by T cells, B cells, and the synchronized interactions of pro-inflammatory cytokines. When naïve T cells differentiate into T (H)17 cells, inflammatory cytokines are produced, which stimulates synovitis [27]. Additionally, citrullination, which is the enzymatic transformation of arginine amino acid molecules to citrulline residue in the connective tissue protein like vimentin. These proteins may trigger an immune response if their forms are drastically changed because the immune system may interpret them as antigens. This causes inflammatory cytokines such as TNF-α, IL-15, IL-1, IL-6, and IL-17 to be produced by macrophages, which has harmful consequences on the afflicted joint [28].

Here in, induction of RA in rats by formaldehyde showed momentous rise of serum TNF-α and IL1-B with significant decreases of serum IL-10. While, administration of monotherapy alone or in combination, produced improvement of these above mentioned inflammatory & Anti-inflammatory markers. According to **Koyama *et al.*** [27], the anti-inflammatory effects of MTX might be due to the participation of adenosine, intrinsic anti-inflammatory factor in arthritis that restricts neutrophils migration to the inflammatory regions and inhibits the generation of leukotriene B4 and interleukin-1. Other researchers have reported that MTX therapy reduced the extent of arthritis by down regulating the production of pro-inflammatory IL-6, TNF-α, and IL-17A cytokines [22].

Our results also are in line with **El-Ghannam *et al.*** [29] who exhibited that LIN has anti-inflammatory activity against colitis rats model and mentioned that the potential mechanism of LIN via reduction of IL-6, TNF-α , and NF-κB p65 in addition to restoration of IL-10, the anti-inflammatory cytokine. Similarly, another author [26] stated that the anti-inflammatory effect of LIN produced by suppressing the pro-inflammatory mediators IL-1β and TNF-α, as well as, inhibiting iNOS and NF-κB in the rat

model of acute kidney damage caused by endotoxin-shock.

In RA, apoptosis may play multiple roles. Even though there are at least 14 caspases, lipid peroxidation and free radicals activate caspase-3, which is necessary for apoptosis**.** While, BCL-2 protein family exhibit anti-apoptotic properties and have been investigated intensely over the past decade for their role in regulating apoptosis [(30)]**.** In our study, arthritic animals displayed significant upsurge in serum caspase-3 and major reduction of serum Bcl-2 while, administration of LIN either alone or combined with half of MTX therapy improved apoptosis markers. These outcomes are in covenant with **Arab *et al.*** [(31)] who documented that LIN treatment exposed clear anti-apoptotic properties by upregulating the expression of Bcl2, the anti-apoptotic protein, and down regulating Bax, the pro-apoptotic protein, in rats with testicular dysfunction that prompted by cadmium.

In this research, induction of RA by formaldehyde led to significant histopathological changes in the form of bone erosion, multiple empty spaces, congested blood vessels and sever cellular infiltration with sever fibrosis. Similar results obtained by **Farrukh *et al.*** [(13)]**.** However, administration of MTX and LIN alone or in combination in this work showed obvious improvement in the joint construction with normal cartilage structure and flattened synovial membrane. Interestingly, the combination group displayed nearly normal joint structure similar to that of the control animals. These results are supported by **Teng *et al.*** [(32)] who found that when treating RA with MTX, it can reduce bone damage brought on by glucocorticoids and inhibit the production of osteoclasts. Also, our results go parallel with the study by some author [(23)] who indicated that linagliptin reduced the histologic injury score in the colon by about 33.4% in addition to attenuation of colonic edema, ulcerations, and wall thickening.

Certain cells, such as endothelial cells, monocytes, vascular smooth muscle cells, fibroblasts and macrophages are stimulated to express COX2 when the body is exposed to pro inflammatory cytokines. COX2 overexpression persuades the production and accumulation of prostaglandins as well as the inflammatory mediators in the injured tissues, which stimulates local inflammation and tissue destruction. Hence, one important indicator of inflammation is believed to be elevated COX 2 expression [(33)]**.**

In the current work, the immune stained sections from the arthritic group presented intense COX-2 immunoreactivity in the majority of the osteocytes. According to **Farrukh *et al.*** [(13)]**,** increased expression of COX-2, which raised the amount of PGE2 in the joint synovial space and this is linked to fluid discharge, inflammation, erythema, pain, and vasodilation. This study exhibited reduced COX-2 immunoreactivity when arthritic animals treated with MTX alone or LIN alone while, combination treatment of LIN and MTX displayed very weak COX-2 immune expression. Correspondingly, **Li *et al.*** [(34)] found that COX-2 level was considerably reduced in MTX-treated rats compared with the RA model group. Also, another author found that macrophages inflammatory protein-2 (MIP-2) and the pro inflammatory marker cyclooxygenase-2 (COX-2) were down-regulated in response to linagliptin. Hence, it attenuated inflammation in the wounds of diabetic mice [(35)]**.**

CONCLUSION

The findings of this study demonstrated that LIN alone or in combination with half of therapeutic dose of MTX has anti-arthritic activity against RA but combination is superior to LIN alone due to synergistic effect of both drugs.

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