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Original article

Evaluation of the Possible Effect of Trimetazidine, Empagliflozin in Comparison with Methotrexate in a Rat Model of Rheumatoid Arthritis

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Abstract

Background: Rheumatoid arthritis (RA) is a chronic, relapsing inflammatory and autoimmune multisystem disease. The main cause of mortality in RA is cardiovascular catastrophes. Methotrexate is considered the most important disease-modifying anti rheumatic drug (DMARD). Trimetazidine is a metabolic modulator-antianginal agent with potent antioxidant, anti-inflammatory, and cytoprotective effects. Empagliflozin is an antidiabetic agent that selectively inhibits (SGLT2) in proximal renal tubules with anti-inflammatory and cardio- renal protective properties. **Aim of the study:** The present study was designed to evaluate the possible effects of trimetazidine or empagliflozin in comparison to methotrexate on adjuvant-induced arthritis model. **Materials and methods** Rats were classified into: Group I: control normal group. Group II: was not treated (diseased group). Group III: was treated with methotrexate. Group IV: was treated with trimetazidine. Group V: was treated with empagliflozin. Group VI: was treated with combination of MTX with trimetazidine. Group VII: was treated with combination of MTX with empagliflozin. Treated groups received drugs for 4 weeks**.** **Results**: Treated groups showed significant improvement in (rheumatoid factor, anti-cyclic citrullinated peptide, tumor necrosis factor alpha, interleukin-6, C-reactive protein, reduced glutathione, cardiac Troponin-I, arthritis score) and significant improvement of the histopathological lesions of the joint and myocardium. A significant reduction in the score was seen in the treated groups at the end of 3rd and 4th weeks, with best results in MTX with empagliflozin combination group. **Conclusion:** All tested drugs alone or in combination showed improvement of parameters of RA with cardioprotective properties.

Key words: Rheumatoid arthritis, Methotrexate, Trimetazidine, Empagliflozin.

Introduction

Rheumatoid arthritis is an immune-mediated chronic inflammatory disorder characterized by pain, swelling and joints destruction. The incidence of RA is increasing affecting 0.5 to 1% of adults worldwide **[1].**

The pathogenesis of the rheumatoid joint involves hyperplasia of the synovial lining cells and mononuclear cell infiltration within the synovium. Moreover, destruction of the cartilage and bone occurs as a consequence of pro-inflammatory cytokines **[2].** The systemic inflammation in RA affects the heart, where it causes myocarditis and ischemic heart disease **[3].**

Main classes of drugs currently used in RA are: NSAIDs, glucocorticoids, non-biologic and biologic DMARDS **[1].**

Methotrexate could be regarded as an important drug not only to control disease activity in RA, but also to reduce cardiovascular risk. One strategy for treatment of RA is the design of drugs that could decrease cardiovascular disease risk by tight control of inflammation **[4].**

Trimetazidine is used as an antianginal agent that acts by blocking beta-oxidation of fatty acids via inhibiting the long-chain 3-acetyl-CoA thiolase. Trimetazidine has been shown to exert potent anti-inflammatory and cytoprotective effects in several experimental models **[5].**

Empagliflozin is an antidiabetic agent that selectively inhibits (SGLT2) in proximal renal tubules leading to increase glucose excretion in urine. Empagliflozin reduces hyperglycemia independently of insulin secretion; therefore, it is not associated with hypoglycemic risk **[6].**

Empagliflozin anti-inflammatory properties were reported in several non-diabetic experimental conditions. Empagliflozin has showed additional benefits in autoimmune diseases through altering T cell immunometabolism and decreased glycolysis in CD4 + T cells **[7,8].**

Aim of work

The present study was designed to evaluate the effects of trimetazidine or empagliflozin mono-therapy in comparison with methotrexate mono-therapy on adjuvant-induced arthritis model. Also, exploring if combination therapy of MTX with TMZ or EMPA can offer some add-on benefits as regard controlling disease activity over MTX mono-therapy.

Materials and method

It is an experimental prospective study conducted during the period from February 2023 to July 2023.

Animals:

The study is carried out on 42 adult male wistar rats obtained from Experimental Animal Breeding Farm, Helwan-Cairo) weighing between 150- 200 g, used for in vivo experiments. They were acclimatized for one week and were caged (6 rat/ cage) in fully ventilated room at room temperature in the pharmacology department, Benha

Faculty of Medicine. Rats were fed a standard chow with water. This study was approved from ethical committee of Benha Faculty of Medicine **(Approval Code: MD 6-12-2022).**

***Drugs and chemicals*:**

Complete Freund's Adjuvant (Sigma-Aldrich Co., USA), Methotrexate (Minapharm Co., Egypt), Trimetazidine (Servier Co., Egypt), Empagliflozin (Boehringer Ingelheim Co., Germany), Carboxy-methyl cellulose (El Nasr Chemicals Co. Egypt), Neutral 10% formaline (El Gomhoria Chemical Co., Egypt), Urethane (Sigma-Aldrich Co., USA), Hematoxylin and eosin (Biostain ready reagents, UK), Anticyclic citrullinated peptide kits (Alpha diagnostic international, USA), Rheumatoid factor kits (CliniLab Company, Egypt) ,Tumor necrosis factor alpha kits (Quantikine TNF-α Immunoassay USA & Canada, R&D Systems, Inc). Interleukin-6 kits (MyBioSource, USA). CRP kits (eBioscience, UK), Reduced Glutathione kits (Biodiagnostic Co., Egypt). Troponin- I kits (BioCheck, USA). Methotrexate was dissolved in distilled water **[10]**. Trimetazidine was suspended in 0. 5% carboxymethyl cellulose **[11].** Empagliflozin was dissolved in distilled water **[12].**

Induction of RA:

Complete Freund's Adjuvant Arthritis was induced by S.C injection of 0.4 ml of CFA in the right hind limb for 12 days in three doses (one dose every four days) **[9].**

Experimental design:

The rats were classified into 7 equal groups (n=6) as follow:

Group (1): non-arthritic control group:

This group received a standard chow and tap water with no medication.

Group (2): rats with adjuvant arthritis group:

This group was injected with (CFA) to induce RA and received no treatment.

Group (3): Rheumatoid arthritis rats treated with Methotrexate group:

This group was treated with methotrexate in a dose of (0.6 mg/kg/week/by gavage) for 4 weeks **[10]** after induction of RA.

Group (4): Rheumatoid arthritis rats treated with Trimetazidine group:

This group was treated with Trimetazidine in a dose of (10 mg/kg/day/by gavage) **[11]** for 4 weeks after induction of RA.

Group (5): Rheumatoid arthritis rats treated with Empagliflozin group:

This group was treated with Empagliflozin in a dose of (10 mg/kg/day/by gavage) **[12]** for 4 weeks after induction of RA**.**

Group (6): Rheumatoid arthritis rats treated with Methotrexate & Trimetazidine combination group:

This group was treated with methotrexate at a dose (0.6 mg/kg/week/by gavage) plus

trimetazidine in a dose of (10 mg/kg/day/by gavage) for 4 weeks after induction of RA.

Group (7): Rheumatoid arthritis rats treated with Methotrexate & Empagliflozin combination group:

This group was treated with methotrexate at a dose (0.6 mg/kg/week/by gavage) plus Empagliflozin in a dose of (10 mg/kg/day/by gavage) for 4 weeks after induction of RA.

Dose selection was based on previously published studies and pilot experiments.

At the end of this study, blood samples were collected from the retro-orbital venous plexus of rats using microcapillary tubes. The blood was collected in clean dry glass centrifuge tubes and incubated at 37° C until clotting, and then centrifuged at 3000 (rpm) for 15 minutes, for separation of serum and stored at -20° C for biochemical analysis **[13].**

Rats were euthanized, hind paws and hearts were removed. They were embedded in paraffin after fixing in formalin solution (10% neutral buffered) for histopathological examination.

Histopathological examination of the joint:

Sections were cut in slices having thickness of 6 um and examined under microscope after hematoxylin-eosin staining for synovium inflammatory cell infiltration and villous hyperplasia **[14].**

Histopathological examination of the cardiac tissue:

Transverse sections (2mm thickness) of the left ventricle wall were stained with hematoxylin and eosin then cardiac sections were examined for the presence of degenerative changes **[15].**

Assessment of arthritis:

a- Arthritis index by a set visual criterion at the end of each week of experiment according to the following criteria: No change = 0, Erythema = 1, Mild swelling = 2, Gross swelling = 3, Gross swelling and deformity = 4 **[16].**

b- Microscopic examination of paw joint stained by H&E stain.

Biochemical assays:

Serum RF **[17].** Serum anti-CCP **[18].** Serum TNF-α **[19].** Serum IL-6 level **[20].** Serum CRP **[21]** were determined by ELISA technique. Serum GSH was determined by using a colorimetric method **[22].** Serum Troponin -1 activity was determined according to Bodor method **[23].**

Statistical analysis:

The collected data were summarized in terms of mean ± Standard Deviation. Comparisons between the different study groups were carried out using (ANOVA) followed by post hoc tests using the Statistical Package for Social Science (SPSS) program, version 26 (Chicago IL

USA, 2000). P-value < 0.05 was considered statistically significant.

Results

Injection of (CFA) resulted in significant increase in serum RF, anti-CCP antibodies, TNF-α, IL-6, CRP and Troponin-I compared to normal group. There was significant decrease in GSH compared to normal group **(Table1)**. There is a significant progressive increase in arthritic score of diseased groups every week compared to the score at 1st day before adjuvant injection **(Table2)**. Joints obtained from diseased group showed inflammatory infiltrates and pannus formation. Also, there was significant increase in histopathological cardiac injury score **(Figure 1,2).**

Regarding the monotherapy treated groups with methotrexate, trimetazidine and empagliflozin, there was significant improvement in serum RF, anti-CCP antibodies, TNF-α, IL-6, CRP, GSH and Troponin-I compared to diseased group. Monotherapy with methotrexate showed the best results **(Table1).**

Regarding arthritis score, a significant reduction in the score was seen in treated groups at the end of 3rd and 4th weeks compared to diseased group. MTX treated group was the best **(Table2).**

Regarding joint histopathology, monotherapy with MTX, Trimetazidine or Empagliflozin showed variable degrees of improvement. MTX treated group showed high degree of improvement **(Figure 1).**

Heart histopathological examination revealed variable degree of improvement in cardiac injury. This improvement was observed in all monotherapy treated groups with best result in empagliflozin group **(Figure 2).**

All combination treated groups leaded to significant improvement in serum RF, Anti-CCP, TNF-α, IL-6, CRP, GSH and Troponin-I compared to diseased group with improvement of arthritic score at the end of 3rd and 4th weeks as well as improvement of the histopathology of joint and heart. Best results were observed in (methotrexate + empagliflozin) combination group **(Table1,2-Figure 1,2).**

Table (1): The effect of methotrexate, trimetazidine, empagliflozin, (MTX+TMZ) and (MTX+EMPA) on (Serum CRP, GSH, TNF-α, IL-6, RF, Anti-CCP and Troponin -I) on experimentally induced RA in rats:

Group Parameter	Control	Diseased	MTX ttt	TMZ ttt	EMPA ttt	MTX+TMZ	MTX+EMPA
CRP (ng/ml)	1.05±0.21	14.32±1.04 a	7.73±0.57 ab	11.53±0.79 abc	9.23±0.59 abcd	3.05±0.49 abcde	2.33±0.53 abcde
GSH (µg/ml)	28.60±0.98	5.42±0.63 a	16.72±0.43 ab	10.82±0.44 abc	14.50±0.99 abcd	24.32±0.93 abcde	25.17±0.59 abcde
TNF-α (Pg/ml)	10.12±0.59	83.67±2.01 a	35.23±0.87 ab	56.43±5.95 abc	37.07±1.58 abd	19.58±1.88 abcde	12.97±1.90 bcde
IL-6 (pg/ml)	23.55±1.92	70.33±2.85 a	35.25±2.36 ab	41.60±1.87 abc	37.75±1.68 abd	29.55±2.43 abcde	25.77±1.05 bcde
RF (IU/ml)	9.47±0.83	49.50±2.38 a	19.27±1.18 ab	28.50±1.74 abc	24.55±0.72 abcd	11.80±0.85 bcde	10.95±0.66 bcde
Anti CCP (U/ml)	7.07±0.65	44.02±2.20 a	15.28±1.32 ab	28.93±0.81 abc	23.07±1.81 abcd	9.55±1.24 bcde	8.55±1.29 bcde
Troponin-I (ng/ml)	0.06±0.04	1.35±0.10 a	1.03±0.10 ab	0.83±0.14 abc	0.57±0.08 abcd	0.37±0.10 abcde	0.09±0.04 bcdef

a: Significant versus control group at p<0. 05 **b:** Significant versus diseased group at p<0. 05

c: Significant versus MTX group at p<0. 05 **d:** Significant versus TMZ group at p<0. 05

e: Significant versus EMPA group at p<0. 05 **f:** Significant versus MTX+ TMZ group at p<0. 05

Table (2): The effect of methotrexate, trimetazidine, empagliflozin, (MTX+TMZ) and (MTX+EMPA) on arthritic score at different times, on experimentally induced RA in rats:

	Control	RA (diseased group)	METHOTREXATE	TRIMETAZIDINE	EMPAGLIFLOZIN	MTX+TMZ	MTX+EMPA
End of 3 doses of CFA	0.0±0.0	2.3±0.46	2±0.36	2±0.56	2±0.34	1.8±0.38	1.7±0.4
End Of 1st w TTT	0.0±0.0	3.5±0.8	2.9±0.5	3±0.5	3±0.88	2.7±0.68	2.6±0.61
End of 2nd w TTT	0.0±0.0	3.5±0.6	2.62±0.6	2.8±0.6	2.73±0.6	2.26±0.4	2.1±0.6
End of 3rd w TTT	0.0±0.0	4±0.67	2.31±0.4 #	2.77±0.5 #	2.56±0.33 #	1.57±0.18 #$%α	1.3±0.24 #$%α
End of 4th w TTT	0.0±0.0	4±0.57	1.8±0.17 #	2.67±0.14 #$	2.34±0.12 #$	1.11±0.18 #$%α	1±0.14 #$%α

#: Significant versus diseased group at p<0. 05 **$:** Significant versus MTX group at p<0. 05

%: Significant versus TMZ group at p<0. 05 **α:** Significant versus EMPA group at p<0. 05

Joint histopathological changes:

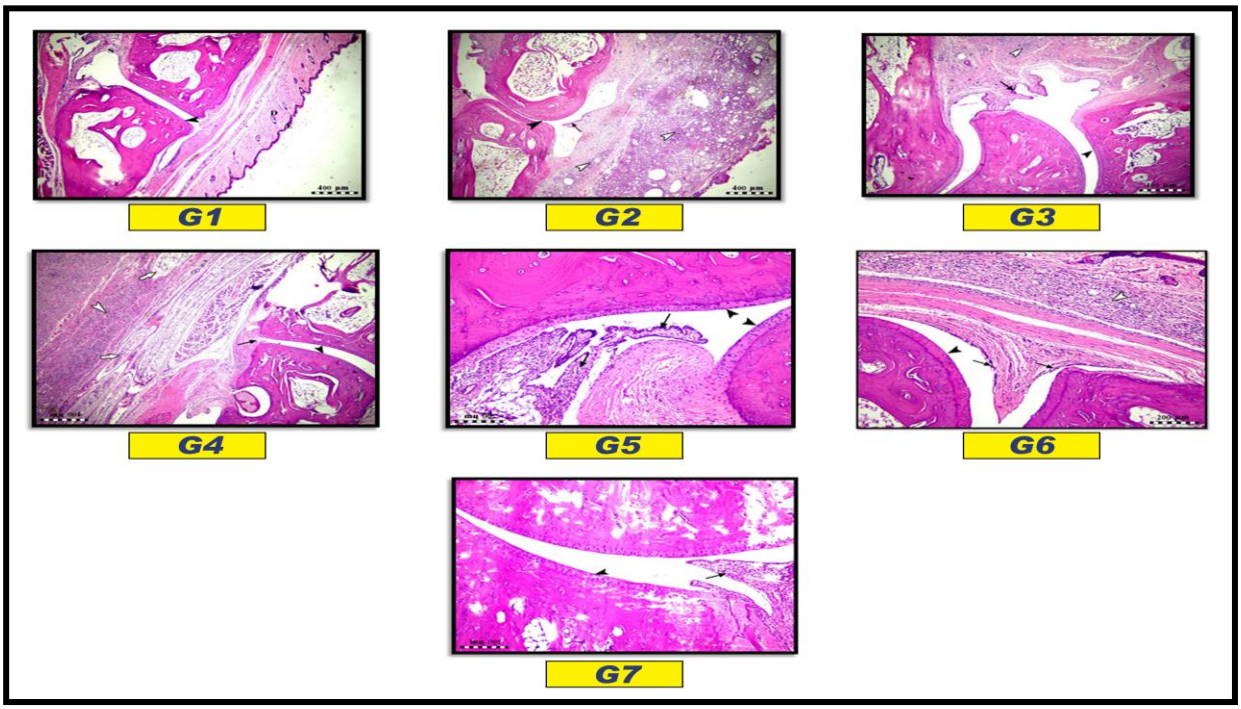

Fig. 1 (G1): photomicrograph of paw of control group showing normal structure of skin, synovium and regular articular surface (H&E x 400).
Fig. 1 (G2): photomicrograph of paw of RA group showing presence of lymphatic nodules, hyperplasia of synovial membrane, tears in the articular cartilage and congestion of blood vessels (H&E x 400).
Fig. 1 (G3): photomicrograph of paw of MTX group showing inflammatory cells infiltration, mild hyperplasia of synovial membrane and apparently intact articular cartilage (H&E x 400).
Fig. 1 (G4): photomicrograph of paw of TMZ group showing moderate lymphocytic aggregation and apparently intact synovium (H&E x 400).
Fig. 1 (G5): photomicrograph of paw of EMPA group showing slight activity of stromal cell with mild hyperplasia of synovial membrane in addition to apparently intact articular cartilage (H&E x 400).
Fig. 1 (G6): photomicrograph of paw of MTX+TMZ group showing mild stromal cell activity and apparently intact articular cartilage (H&E x 200).
Fig. 1 (G7): photomicrograph of paw of MTX+EMPA treated group showing normal stromal cell activity, mild lymphocytic aggregation with normal synovial membrane (H&E x 400).

Heart histopathological changes:

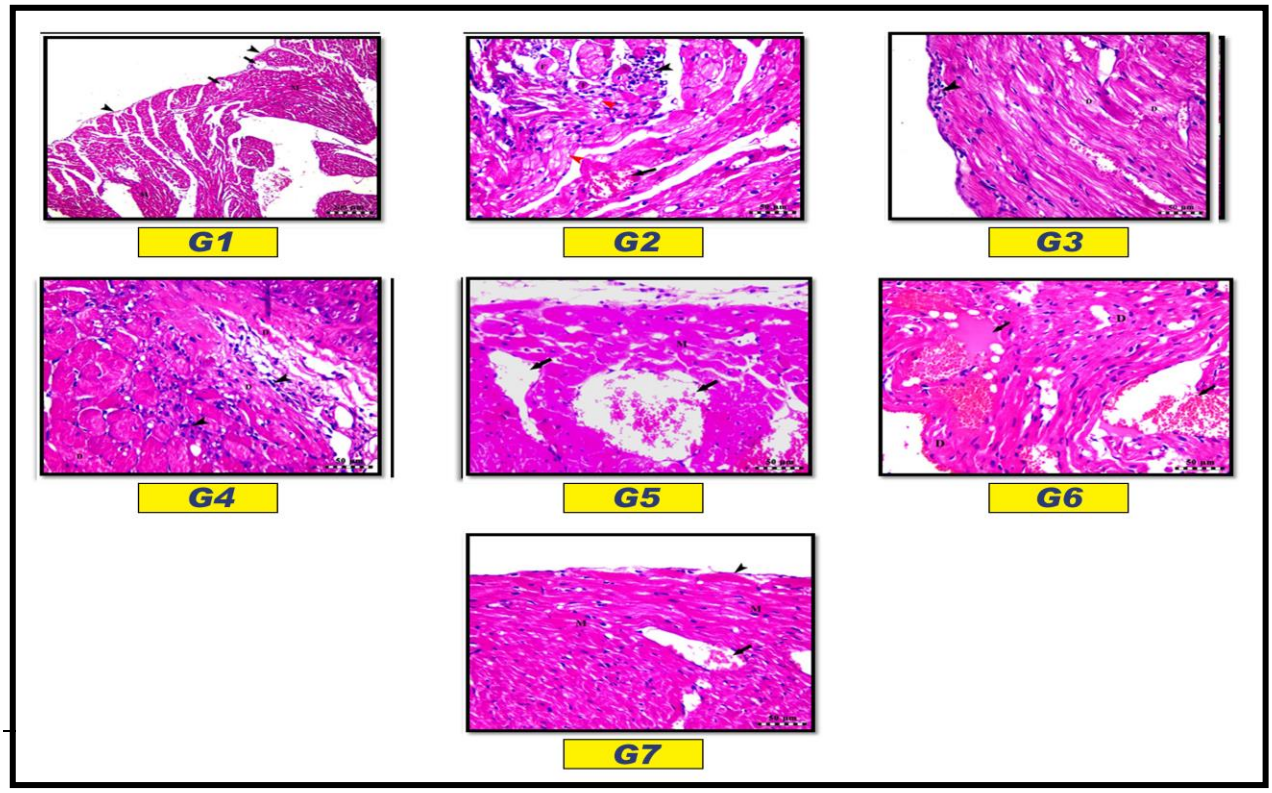

Fig. 2 (G1): photomicrograph of heart of control group showing normal structure the cardiac muscles (M) arranged in cross and longitudinal sections covered by epicardium (H&E x 200).
Fig. 2 (G2): photomicrograph of RA group showing Aschoff nodule in addition to fibrinoid necrosis (F), vacuolar degeneration of myocardium (H&E x 50).
Fig. 2 (G3): photomicrograph of MTX group showing small aggregation of inflammatory infiltration in addition to loss of acidophilia in some cardiac muscle fibers indicating presence of degenerative changes (H&E x 50).
Fig. 2 (G4): photomicrograph of TMZ group showing aggregation of inflammatory cells infiltration in addition to degenerative changes of some myocardial fibers (D) (H&E x 50).
Fig. 2 (G5): photomicrograph of EMPA group showing mild congestion of blood vessels with apparently intact myocardial fibers (M) (H&E x 50).
Fig. 2 (G6): photomicrograph of MTX+TMZ group showing severe congestion of cardiac blood vessels with mild myocardial degeneration (D) (H&E x 50).
Fig. 2 (G7): photomicrograph of heart of MTX+EMPA group showing normal structure of the myocardium (M) covered by epicardium (H&E x 50).

Discussion

The present work was designed to evaluate the possible anti-inflammatory and cardioprotective effects of (Trimetazidine and Empagliflozin) alone or in combination with methotrexate in (RA) induced experimentally in rats by S.C of (CFA) in right hind limb, for 4 weeks treatment through evaluation of their effects on (RF) , (anti-CCP), (TNF-α) , (IL-6), (CRP) , (GSH)

(CTn-I), arthritis score and histopathological changes of joint & myocardium.

The choice of (CFA) because it resembles rheumatoid arthritis in pathology and mimics multiple aspects of cardiac abnormalities observed in RA**[24].**

The data of the present work revealed that s. c injection of CFA resulted in increase in serum RF, anti-CCP, TNF-α, il-6, CRP, CTn-I, arthritic score, decrease in serum GSH and affected the pathology of the joint and myocardium. These finding are similar to the observations of other study **[25]** which proved that injection of CFA led to significant elevation of serum RF, anti-CCP, TNF-α, IL-6, CRP and decrease serum total anti-oxidant capacity compared to normal control group. There was a significant increase of the arthritic score in the CFA group. The results of joint histopathology revealed inflammatory infiltrations and synovial hyperplasia in arthritic rats in contrast to controls.

These results are in agreement with previous study **[26]** reported that injection of CFA leads to significant rise in serum (CTn-I) in CFA rats compared with normal control group.

Methotrexate significantly improved all the tested parameters, arthritic score and histopathology of the joints and hearts. The results of this study are in agreement with the observations of other study **[27]** which reported that MTX treatment after induction of RA by CFA, induced a highly significant decrease in serum RF, anti-CCP, TNF-α, CRP and induced a highly significant increase in blood GSH compared with arthritic rats. Also, MTX significantly improve arthritic score and histopathology of the joints.

Methotrexate can resist several signaling pathways, including the (JAK- STAT) and (NF-κB) which are crucial for the inflammatory response in RA **[28]**

Also, a previous study was in line with our results as MTX administration decreased cardiac troponin in patients with AMI [**29]**.

Methotrexate can reduce cardiac troponin by suppress systemic inflammation, a key driver of cardiovascular complications in RA [**30]**.

Regarding monotherapy with trimetazidine, it showed significant improvement of serum RF, anti-CCP, TNF-α, il-6, CRP, GSH and troponin I compared to diseased group. This group showed improvement of the arthritic score at the end of 3rd and 4th weeks. Also, TMZ improved histopathology of the joint and myocardium.

Our study is in line with a recent study which showed significant decrease in serum anti- CCP and serum RF in trimetazidine treated arthritic rats **[31].**

The immunomodulatory effect of trimetazidine can be attributed to blocking the differentiation of T cells and binding of CD4+antibodies to them **[32].**

In addition, serum TNF-α & IL-6 were significantly reduced by trimetazidine treatment in rats with cardiotoxicity, as reported in our study **[11].** Moreover,

administration of trimetazidine significantly decreased serum CRP in arthritic rats as proved by a study of other researchers **[31]**.

Trimetazidine mitigate inflammatory responses by inhibiting the TLR4/NF-KB pathway with subsequent suppression of CRP and cytokines production **[33]**.

Our study is in line with a previous study showed that trimetazidine has increased GSH significantly in rats **[34].**

The antioxidant effect of trimetazidine can be explained by activation of (Nrf2), a transcription factor pivotal in cellular defense against oxidative stress **[35].**

According to arthritis score and joint histopathology, trimetazidine showed significant, improvement of arthritis score especially at 3rd and 4th weeks and decreased inflammatory infiltrations in joint histopathology. This is in line with a previous study proved the beneficial effects of TMZ in arthritic rats **[31].**

Significant improvement of serum CTn-I and improved myocardium histopathological changes by trimetazidine were observed in previous study investigating cardio-protective effects of TMZ **[11]**

The cardioprotective effect of trimetazidine can be explained by decreasing myocardial cell acidosis and calcium overload and elevation of the antioxidant capacity **[32]**.

Concerned empagliflozin monotherapy, our study showed significant improvement of serum RF, anti-CCP, TNF-α, IL-6, CRP, GSH and Troponin I, compared to diseased group. For arthritic score, this group showed improvement of the score at the end of 3rd and 4th weeks. Also, it improved histopathology of the joint and heart.

Recent studies reported that empagliflozin mediated modulation of mitochondrial function and immunometabolism via decreased T cell glycolysis; may contribute to the immunomodulatory benefits of this drug in autoimmune diseases **[8]**.

Our results are in consistence with a recent study reported that empagliflozin significantly reduced expression of TNFα and IL-6 in Lipopolysaccharide activated microglia in rats **[12]**.

Also, these data are in harmony with a study that showed the effect of empagliflozin in reduction of CRP in AMI rats **[36]**.

Empagliflozin decreases the expression of (NF-κB) and activates AMPK, so inhibits the production of pro-inflammatory cytokines with subsequent reduction of CRP **[37]**.

In addition, the level of serum GSH was significantly increased by empagliflozin treatment. This is supported by a study of the reno- protective effects of empagliflozin in diabetic rats **[38]**.

The antioxidant effect of empagliflozin can be explained by reduction in mitochondrial ROS production and reduction of (HIF-1α) expression **[39]**.

Improvement in arthritic score and improved joint histopathology by empagliflozin are supported by a recent study reported

chondroprotective effect of empagliflozin via inhibition of (NF-κB) **[40].**

Empagliflozin treatment resulted in significant decrease in Troponin I and improved cardiac injury in histopathological examination. These findings are in line with previous study proved the cardioprotective effect of empagliflozin **[41]**. Empagliflozin improves cardiac outcomes by reducing cardiac inflammation via AMPK activation **[42].**

Regarding combination groups, they produced significant improvement of all parameters, arthritic score and histopathological changes of the joint and heart. They were better than monotherapy treated groups with best results in (methotrexate + empagliflozin) combination group.

Regarding (methotrexate + trimetazidine) combination and in consistence with our study**,** recent study highlighted that administration of trimetazidine with methotrexate combination can offer some add-on benefits over MTX mono-therapy in arthritic rat **[31]**.

Regarding (methotrexate + empagliflozin) combination, our study was in harmony with a recent study highlighted the synergistic administration of empagliflozin with methotrexate as a potential approach to reduce adverse effects while improving therapeutic efficacy in RA treatment **[43].**

Conclusion

Trimetazidine and empagliflozin have antiarthritic and cardioprotective effects in adjuvant arthritis. The combination of methotrexate with trimetazidine or empagliflozin in the treatment of RA provide enhanced anti-inflammatory effects and potential protection against methotrexate high dose-induced toxicity.

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