Histopathological evaluation of docetaxel effects in treatment of rheumatoid arthritis induced in rat model

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Abstract:

Rheumatoid arthritis is an immune-mediated condition that affects synovial joints. Synovial tissue, cartilage, bone, and less frequently extra-articular structures which in turn experience inflammatory changes. Paclitaxel's semi-synthetic equivalent, docetaxel, is an anti-neoplastic drug. Methotrexate is a treatment for early RA and may have a mildly negative impact on peptidyl arginine deiminase type 4 fluorescence test. However, 30% of patients fail to complete treatment within the first year due to resistance or side effects. The synovial membrane of Rheumatoid arthritis patient infiltrated with macrophages and neutrophils that express peptidyl arginine deiminase type 4 which their effect in rheumatoid arthritis pathogenesis lies in the generation of citrullinated neoepitopes that are Anti cyclic citrullinated peptide antibodies-targeted.

The purpose of this study: was to assess the anti-inflammatory effects of docetaxel and methotrexate on the joint structure.

Methods: Five groups of eight rats were formed from the 40 male Wister rats. Complete Freund's adjuvant was injected subcutaneously into rats to induce the disease. The first group is control group which was the only group consists of (healthy untreated) rats. Second group was received complete Freund's adjuvant. 0.5ml of ordinary saline was intraperitoneally administered to both the control and induction groups. Based on a preliminary experiment, the third group was given intraperitoneally 1 mg/kg/on alternative day docetaxel. The fourth group was given intraperitoneally 1 mg/kg/week of Methotrexate. Fifth group was given a half dose of both Methotrexate and docetaxel concurrently. Arthritis index was measured and Knee joint was histopathological examined.

Results: significant Arthritis Index decrease in docetaxel group ($p \leq 0.05$). Significant lowering Histometric scoring ($p \leq 0.05$) in docetaxel, and Methotrexate group (cellular hyperplasia, formation of granulation tissue, infiltration of leukocytes, destroying of cartilage and intensity of erosion & Articular cartilage thickness) level in rats induced arthritis.

Conclusion: This study showed that docetaxel may have anti-arthritic effects through their significant lowering Histometric scoring($p \leq 0.05$).

Key words: Rheumatoid arthritis, docetaxel, Histopathology, Arthritis index

التقييم النسيجي المرضي لتأثيرات الدوسيتاكسيل في علاج التهاب المفاصل الروماتويدي المستحث في نموذج الجرذان البيضاء

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الخلاصة:
التهاب المفاصل الروماتويدي هو اضطراب مناعي يؤثر على المفاصل الزليليّة، النسيج الزليلي، والغضاريف، والعظام، اما الاجزاء خارج المفصل في بعض الأحيان تعاني من تغيرات التهابية. اما الدوسيتاكسيل فهو المكافئ شبه الاصطناعي لباكليتاكسيل، وهو دواء مضاد للأورام. في حين ان الميثوتريكسات هو دواء يمكن استخدامه في علاج التهاب المفاصل الروماتويدي المبكر وقد يكون له تأثير سلبي معتدل على ببتيدل أرجينين ديميناز النوع الرابع بالفحص الفلوري. ويحتوي الغشاء الزليلي للمفاصل الروماتويدية على الضامات والعدلات التي تفرز ببتيدل أرجينين ديميناز النوع الرابع. تكمن كفاءتها في توليد المناظير الجديدة المحتوية على سيترولين والتييتم استهدافه بواسطة الأجسام المضادة الببتيدية السيترولينية الحلقية المضادة. الغرض من هذه الدراسة هو تقييم التأثيرات المضادة للالتهابات للدوسيتاكسيل والميثوتريكسات على بنية المفصل.
الطريقة: تم تشكيل خمس مجموعات من ثمانية جرذان مختبرية من ٤٠ ذكور جرذان الوستر. يتم حقن مساعد فرويند الكامل تحت الجلد في الجرذان المعرضة للإصابة بالمرض. تتكون المجموعة الضابطة من الجرذان البيضاء الغير محفزة. مع المجموعة الثانية، تم تقديم مساعد فرويند الكامل. تم إعطاء ٠,٥ مل من المحلول الملحي العادي داخل الصفاق لكل من مجموعتي التحكم والتحريض. بناءً على تجربة أولية، أعطيت المجموعة الثالثة ١ مجم / كجم / كل يومين. من الدوسيتاكسيل بعد إحداثه بواسطة مساعد فرويند الكامل. تحرض المجموعة الرابعة وتعطى داخل الصفاق ١ ملجم / كجم / أسبوع من الميثوتريكسات. المجموعة الخامسة تعطى نصف جرعة من كل من الميثوتريكسات والدوسيتاكسيل بشكل متزامن. تم تطبيق التشريح المرضي وقياس مؤشر التهاب المفاصل.
النتائج: انخفاض كبير في مؤشر التهاب المفاصل في مجموعة الدوسيتاكسيل ($p \leq 0.05$). انخفاض ملحوظ في درجات قياس النسيج ($p \leq 0.05$) في مجموعة الدوسيتاكسيل ومجموعة الميثوتريكسات (تضخم خلوي، تكوين الأنسجة الحبيبية، ارتشاح الكريات البيض، تدمير الغضروف وشدة التآكل وسماكة الغضروف المفصلي) في التهاب المفاصل الناجم عن الفئران.
الخلاصة: أظهرت هذه الدراسة أن مادة الدوسيتاكسيل قد يكون لها معاداة حسابية من خلال انخفاض درجاتها النسيجية بشكل ملحوظ ($p \leq 0.05$).

الكلمات المفتاحية: التهاب المفاصل الروماتويدي، الدوسيتاكسيل، علم أمراض الأنسجة، مؤشر التهاب المفاصل

Introduction

Rheumatoid arthritis (RA) is a prevalent inflammatory immune-mediated disease that mostly damages synovial joints. It is characterized by joint immune cell infiltration[1], which is characterized by inflammatory changes in synovial tissue, cartilage, and bone, as well as extra-articular structures but less commonly[2]. An increased risk of cardiovascular disease and a shorter life expectancy is also linked to RA [3]. It has a global yearly incidence and prevalence rate of 3 cases per 10,000 persons and 1%, respectively[4].The prevalence of RA in Babylon is about 3% in Iraq[5].

Citrullination is a post-translational modification performed by the calcium-dependent peptidyl-arginine-deiminase (PAD), which converts positively charged arginine to neutral citrulline[6]. citrulline refers to the dysregulated synthesis of citrullinated proteins in RA joints. Protein citrullination is a key step in the autoimmune response[7]. Failure of central tolerance causes autoimmunity[8].

Macrophages start and promote rheumatoid arthritis pathogenesis as innate immune response[9]. These cells are key producers of the cytokines, chemokines, and degradative enzymes that cause joint inflammation and eventually contribute to the breakdown of cartilage and bone. In addition, macrophages and their products are believed to be engaged in synovial angiogenesis, which plays a crucial role in the pathogenesis of rheumatoid arthritis[9]. They release pro-inflammatory cytokines in RA patients' joints, including TNF- α, IL-1β, IL-8, IL-15, IL-18, and MIF[10]. While Neutrophils are the most common immune cells in RA-inflamed joints, and their ability to create neutrophil extracellular traps (NETs) plays a role in RA pathogenesis through autoantigen generation and FLS activation[11]. Neutrophils have the PADI4 enzyme that citrullinates arginine[12]. Activated

neutrophils release immunological mediators like IL-1β, IL-6, IL-12, TGF-β, and TNF-α, causing acute and chronic inflammation[13].Cytokines are endogenous peptides with high potency and pleiotropy that are produced by various cell types[14].

Adaptive immune response, synovial B cells produce inflammatory cytokines IL-1β, IL-6, IL-12, and TNF-α according to single-cell RNA sequencing[15].

Docetaxel (DTX) is a semisynthetic version of Paclitaxel, a taxoid anti-cancer drug[16]. Docetaxel is four times as antiangiogenic as paclitaxel[17]. Previous studies found that reduction in VEGF, TNF-α, and IL-1β levels in the paclitaxel (PTX) group compared to the control group[18]. Another article mentioned reversible PAD inhibitors (e.g., taxol, minocycline, and streptomycin)[19]

Docetaxel-cisplatin or docetaxel-carboplatin may have anti-arthritic effects, according to a clinical assessment[20,21].

Methotrexate (MTX) is the conventional synthetic disease modifying antirheumatic drugs csDMARD for early RA management [22], But due to its side effects or ineffectiveness, 30% of people taking methotrexate stop their treatment within the first year[23]. it had intermediate effects on PAD4 fluorescence[24]. therefore, MTX should be given a strict comparison to DTX and evaluates its PAD4 inhibitory effect.

Peptidyl-arginine-deiminase type 4 is expressed in macrophages and neutrophils in RA synovial membrane. Their effectiveness is in producing ACPA-targeted citrullinated neoepitopes[25]. Which suggests PAD4 ELISA kit could help diagnose RA.

Aim and objective

To assess the anti-inflammatory effects of docetaxel and methotrexate on the joint structure in rheumatoid arthritis.

Material and methods

Animals

Forty male Wistar rats 12-to14-week-old weighing 200-250 g were purchased from the University of Tikrit's College of Veterinary Medicine. The temperature was maintained at 25°C, and an artificial light unit was used to create a light/dark cycle for the animals' comfort. In the animal house at Mustansiriyah University's College of Pharmacy, the animals have unrestricted access to food and water. It was only after receiving approval from the college of pharmacy's ethics committee that this investigation could begin. This research started in November of 2021 and finished up in May of 2022.

Forty male wister rats were divided into five groups of eight. Normal rats form the control group. Second group is induction with Complete Freud's adjuvant (CFA) which is subcutaneously injected into rats to start the disease at the tail base or between the hind paws Producing rats' swollen joints contain activated T cells[26]. Pathogenesis involves TNF-α, IL-1β, IL-21, and IL-17[27]. Both the control and induction groups received 0.5ml of normal saline intraperitonially. The third group is induced by CFA and given 1 mg/kg/on alternative day DTX based on preliminary experiment. Fourth group was induced, then given 1mg/kg/week MTX intraperitoneally[28]. The fifth group was the combination group that's induced then treated with MTX and DTX in half doses

On days 0 and 10, animals in the induction, DTX, MTX, and DTX+MTX groups received (1.2 and 0.4) ml of CFA in their tail bases. These animals were cared for, and on day 12, conventional treatments Docetaxel and MTX were started till day 33.

Clinical assessment

Arthritis Index

A four-point scale was used to assess the objective aspects of RA in each of the five groups on the day before the end of the experiment after CFA immunization, indicated Clinical finding in (score), No swelling or erythema (0), Slightly swelling

and/ or erythema (1), Low-grade swelling and/or erythema (2), Pronounced swelling with joint movement limitation (3), Massive swelling with joint rigidity (4)[29].

Histopathological Processing

At day 35, cartilage, muscle, synovial membrane, and fluid were surgically removed from rat knee joints. Tissue samples were be frozen at (-10) degrees Celsius and protected from light[30]. After that, the knee joints were subjected to the processing processes listed below[31].

a) After surgical excision, materials were stored in 10% formalin to inactivate enzymes. 48-hour process.

b) Soaking samples in 10% formic acid for 48 hours softens them. This allows osseous samples to be cut[32,33].

c) In this step, samples were sliced to the proper size and portion for the next step.

d) After dehydration, samples were stored overnight in pure alcohol (50, 70, 90, and 100%). Dehydrating samples removes water.

e) Infiltration uses melted paraffin. Molds were filled with molten paraffin to create wax blocks. These blocks were frozen overnight at -20°C.

f) Using a semi-automated rotary microtome, wax blocks were trimmed to 5 mm. These parts were submerged in 40°C water to relax tissues.

g) Dewaxing: Wax was eliminated using a 55° C oven for 20 minutes.

h) Rehydration fights dehydration. The samples were stored in 55oC xylene for five minutes, then 20 to 25oC for five minutes. After two hours, 100, 90, 70, and 50% alcohol were used, then distilled water washed.

i) Haematoxylin was applied for 3 to 5 minutes, then washed with distilled water. Second, apply Eosin 1% stain for 1 to 2 minutes, then wash with distilled water.

j) Protecting our slides with DPX cover slides (dibutyl phthalate polystyrene xylene).

k) Pathologist used a light microscope to examine our slides.

l) Using semiquantitative grading systems (normal joint, or minimal, mild, moderate, or severe illness), an expert pathologist can quickly collect histopathologic data including Synovial lining hyperplasia & changes, Granulation tissue, Infiltration of leukocytes, destroyed cartilage, and Intensity of tissue erosion, which was graded by computer[34,35].

Statistical analysis

The data were presented in the form of means ± standard deviation (M ± STDEV). Data was analyzed with SPSS-20.0. To analyze various means, ANOVA and the post-hoc Tukey test were used. *P*-values equal or less than 0.05 are statistically significant.

Kruskal-Wallis and Mann-Whitney tests were used for non-parametric statistical analysis of nominal data[36].

Results

Arthritis Index of Rheumatoid Arthritis

Arthritis Index (AI) at the end of experiment showed significant increase in induction group compared with control ($p \leq 0.05$). while DTX group showed significant decrease ($p \leq 0.05$) compared with induction group while both MTX and DTX+MTX groups showed slightly decreased as shown in table (١).

Table (1): mean rank of arthritis.

Groups	Number of the lab. Animals	Mean Rank of arthritis index*
Control	8	6.56 a
Induction	8	30.31 b
DTX	8	16.00 c
MTX	8	22.38 b c
DTX+MTX	8	27.25 b

* Data represent mean of rank, test statistics Kruskal Wallis: Asymp. Significant = 0.000
DTX= docetaxel, MTX= methotrexate Different lowercase letters indicate significant differences between groups *($p \leq 0.05$)*

Control group

Histopathological histomicrograph showed normal appearance of articular surfaces of femorotibial, tibiofibular joint and patellar joint, ligaments, meniscus and synovium tissue.as in figure (1).

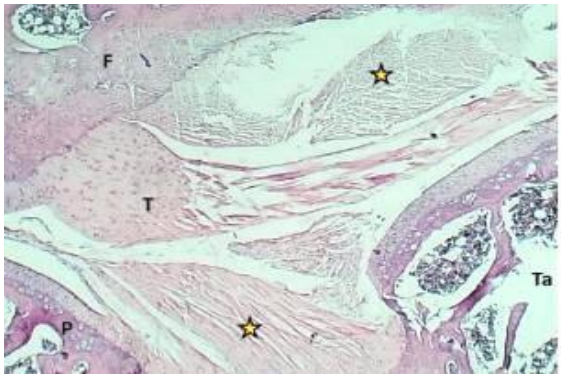

Figure (1-a): section of knee joint (Control group) showed: normal appearance of femur articular surface (F), tibial articular surfaces (Ta), fibular articular surface (P), patellar ligament (T) &. H&E stain.40x.

Figure (1-b): section of knee joint (Control group) showed: normal appearance of femur articular surface (F), tibial articular surfaces (T), meniscus (M).H&E stain.100x.

Induction group

Histopathological figures of knee joint (induction group) showed marked arthritis which characterized by thickening of femorotibial articular surface, enlargement of articular space with marked erosion of articular matrix and damage of chondrocytes. The synovium showed marked formation of granulation tissue formation within edema, the granulation tissue was replacement the damaged cartilage in joint associated with marked angiogenesis. As in figure (2).

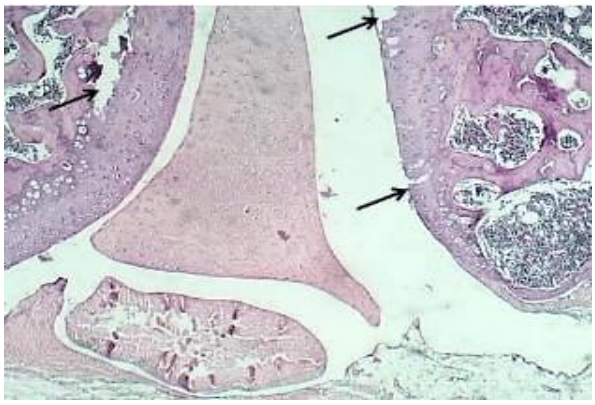

Figure (2-a): section of knee joint (Induction group) showed: thickening of femorotibial articular surface, enlargement of articular space with marked erosion of articular surfaces (arrows) H&E stain.40x.

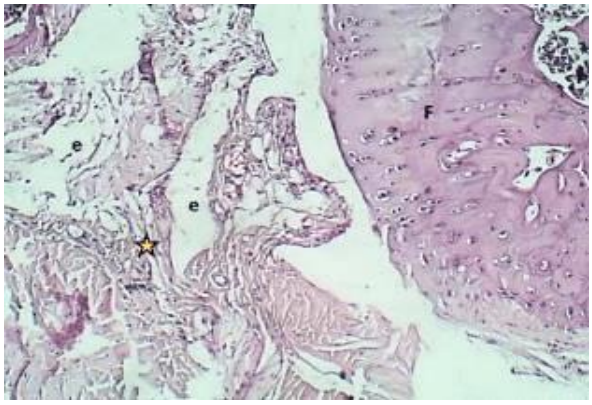

Figure (2-b): section of knee joint (Induction group) showed: granulation tissue formation (Asterisk) within edema (e) at the synovium. H&E stain.100x.

Docetaxel group

In comparison to control group, the histomicrograph of knee joint similar that of control group that showed normal patella and patellar ligaments, normal articular surface appearance, thickness, normal synovial space and tissue, no evidence of inflammatory infiltration and damage. As in figure (3).

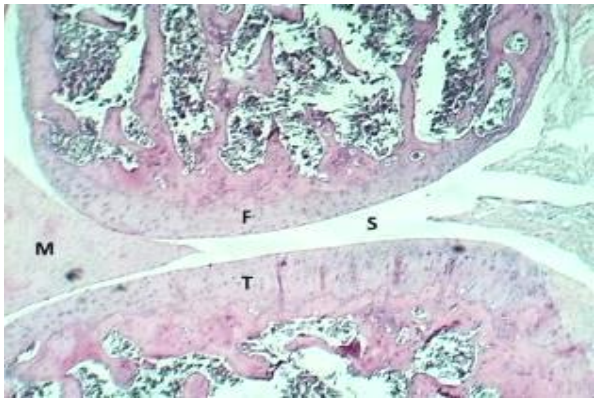

Figure (3-a): section of knee joint (DTX group) showed: normal appearance of femoral articular surface (F), tibial surface (T), meniscus (M) & joint space (S). H&E stain.40x.

Figure (3-b): section of knee joint (DTX group) showed: normal appearance of femoral articular surface (F), fibular articular surface (Fi) & joint space (S). H&E stain.100x.

Methotrexate group

In comparison with induction group, the figures of knee joint showed normal patella and patellar ligaments, marked thinning of femoral condyles articular surfaces, widening of synovial space and joint pad, normal patella and synovial tissue, there were no evidence of inflammatory infiltration and articular damage. As in figure (4).

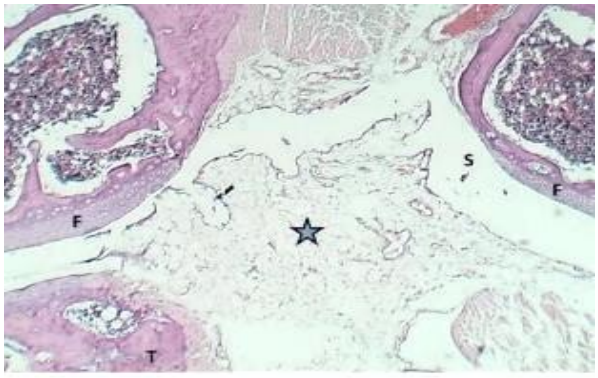

Figure (4-a): section of knee joint (MTX group) showed: widening of joint space (S) and joint pad (asterisk), thinning of femur articular surfaces in both condyles (F), tibial articular surface (T). H&E stain.40x.

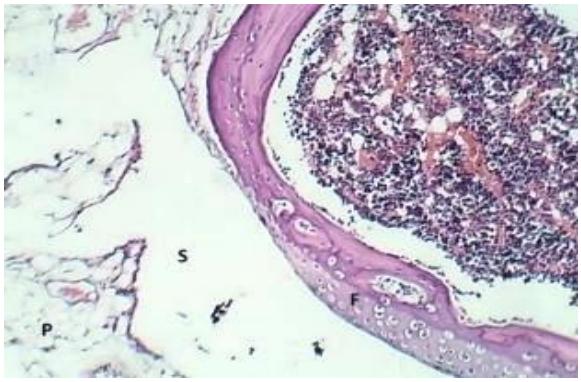

Figure (4-b): section of knee joint (MTX group) showed: normal joint space (S), joint pad (P), thinning of femur articular surface (F). H&E stain.100x.

Combination of Docetaxel + Methotrexate treated group

In comparison with induction group, the histomicrograph of knee joint showed normal meniscus, congestion of blood vessels and degeneration of collagen bundle within synovium, mild erosion of femoral and tibial articular surfaces. Other histomicrograph revealed mild erosion of femoral and patellar articular surfaces with massive destruction of tibial articular surface. As in figure (5).

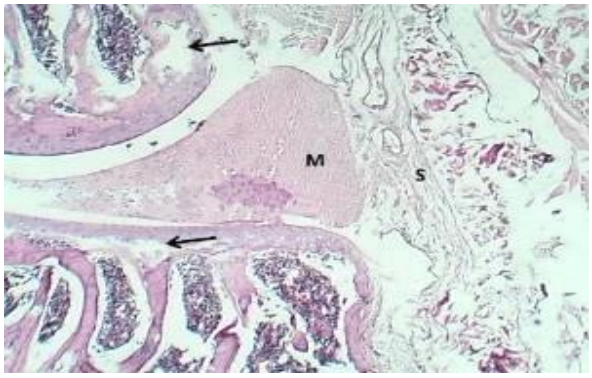

Figure (5-a): section of knee joint (DTX+MTX group) showed: normal meniscus (M), congestion of blood vessels and degeneration of collagen bundle within synovium (S), and mild erosion of femoral and tibial articular surfaces (Arrows) H&E stain.40x.

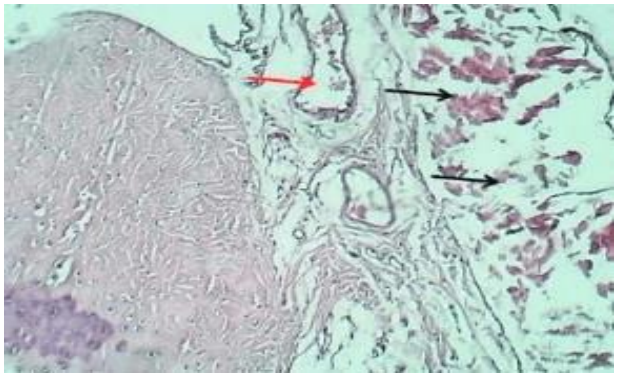

Figure (5-b): section of knee joint (DTX+MTX group) showed: normal meniscus, congestion of blood vessels (Red arrow), and degeneration of collagen bundle within synovium (Black arrows). H&E stain.100x.

Histopathology scoring among studied groups

Histomorphometric scoring of cellular hyperplasia, formation of granulation tissue, infiltration of leukocytes, destroying of cartilage and intensity of erosion & Articular cartilage thickness are applied. Such scoring system was graded as (absent, mild, moderate & sever) and the intensity from (absent, weak, moderate & sever).

Regarding the articular cartilage thickness, the induction group (188.82 ± 4.38) showed increase thickness compared with control group (107.25 ±13.22) with a significant difference ($P \leq 0.05$). DTX group (113.75 ± 6.01) showed non-significant difference ($P=0.365$) compared

with control. In addition, DTX+MTX group (99.69 ± 2.13) & MTX (74.74 ± 3.58) showed non-significant difference *(P=0.22)* & with significant difference (*P ≤0.05)* compared with control.

The data histopathological scorings were not distributed according to the normal curve, so it's used a nonparametric statistical analysis (mean rank), namely the Kruskal–Walli's test, and Mann-Whitney Test to determine significance among groups as shown in table (2).

Table (2): non-parametric test of the histometric scoring of the studied groups

Parameters / Groups	Synovial lining hyperplasia & changes	Granulation tissue	Infiltration of leukocytes	Destroyed cartilage	Intensity of tissue erosion	Articular cartilage thickness /µm
	Kruskal-Walli's test					
	Asymp. Sig.					
	0.204	0.001	0.005	0.001	0.001	
Control	15.75 a	15.69 a	15.50 a	11.50 a	13.31 a	107.25 ±13.22 ac
Induction	22.88 a	35.38 b	30.75 b	34.38 b	35.13 b	188.82 ± 4.38 b
DTX	15.75 a	17.88 a	17.94 a	13.50 a	13.31 a	113.75 ± 6.01 a
MTX	25.25 a	15.69 a	20.38 a	17.50 a c	15.13 a	74.74 ± 3.58 d
DTX+MTX	22.88 a	17.88 a	17.94 a	25.63 c	25.63 c	99.69 ± 2.13 c

Data represent mean of rank

Data are mentioned as means± STDEV (STDEV: standard deviation)

DTX= docetaxel, MTX= methotrexate

Different lowercase letters indicate significant differences between groups *(P ≤ 0.05)* which was done by Mann-Whitney test

The current study showed that there was non-significant difference between all groups in term of Synovial lining hyperplasia & changes. Which the mean rank of control (15.75) showed a non-significant (*P=0.223*) compared with induction (22.88), also DTX (15.75), MTX (25.25) and DTX+MTX (22.88) showed non-significant *(P=0.223) (P=0.817) (P=0.908)* respectively.

In term of Granulation tissue, the mean rank of control group (15.69) showed a significant difference *(P≤0.05)* compared with induction group (35.38). mean rank of DTX group (17.88), MTX group (15.69) and DTX+MTX (17.88) showed a significant difference *(P≤0.05)* compared with induction group.

Regarding the infiltration of leukocytes, the mean rank of control group (15.50) showed a significant difference *(P≤0.05)* compared with induction group (30.75). mean rank of DTX group (17.94), MTX group (20.38) and DTX+MTX (17.94) showed a significant difference *(P≤0.05)* compared with induction group.

In term of Destroyed cartilage, the mean rank of control group (11.50) showed a significant difference *(P≤0.05)* compared with induction group (34.38). mean rank of DTX group (13.50), MTX group (17.50) and DTX+MTX (25.63) showed a significant difference *(P≤0.05)* compared with induction group. Both the mean rank of DTX group and MTX group showed non-significant difference *(P=0.317) (P=0.063)* respectively compared with control group, while DTX+MTX group showed significant difference *(P≤0.05)* compared with control group.

Moreover, intensity of tissue erosion, the mean rank of control group (13.31) showed a significant difference *(P≤0.05)* compared with induction group (35.13). mean rank of DTX group (13.31), MTX group (15.13) and DTX+MTX (25.63) showed a significant difference *(P≤0.05)* compared with induction group. as shown in table (2)

Discussion

Arthritis Index of Rheumatoid Arthritis

Both DTX and induction groups have different arthritis indices (AI). The control group's AI rank was the lowest and the induction group was the highest. All treatment groups had a lower mean rank than the AI group, but to varying degrees. Match with a previous study, when PTX group & MTX group had a lower arthritis index significantly *(P≤0.05)* than the induction group[18].

The effect of Docetaxel, methotrexate and their combination on knee histopathological changes

Compared to the control group, rats induced with Complete Freud adjuvant for rheumatoid arthritis showed significant arthritis changes *(P≤0.05)*, as evidenced by thickening of the femorotibial articular surface, infiltration of leukocytes, and destruction of the chondrocytes and articular matrix. Furthermore, granulation tissue and angiogenesis in the edematous synovium indicate that CFA negatively affected knee tissue architecture. These results matched earlier studies showing CFA caused inflammation and pannus formation.[37]. Such findings were also consistent with a recent study done by Akhter and his coworkers (2022)[38].

Histopathological findings in the DTX group showed a therapeutic effect against CFA-induced inflammation and damage. This group's patella and patellar ligaments, articular surface, thickness, and synovial space are all normal, with almost no inflammatory infiltration or pannus formation. In a previous study, PTX and MTX reduced inflammatory infiltration, pannus development, and joint structure changes. The PTX group's mean score was lower than the Induction group's[18].

Moreover, synovial space, patella, and synovial tissue were normal in the MTX group. Ahmed and his colleagues (2022) & Anchi and his colleagues (2022). Both studies found histopathological differences between MTX and induction groups.[39,40].

In addition, the combination group had normal meniscus, congested blood vessels, and minor articular surface erosion. Sheng and colleagues (2020) found that PTX 3.5mg/kg showed a largely intact synovial structure, infrequent inflammatory cell infiltration, no edema or congestion, and normal blood vessel density. These results showed that PTX and MTX both alone or in combination improved the histology of induced arthritis rats, with PTX at 3.5 mg/kg having the most impact[41]. The therapeutic impact of DTX on RA induced in rats by histopathological examination gave a promising future for DTX to be used in RA.

Conclusion

Current study showed that DTX may have anti-arthritic through their significant lowering Histomorphometric scoring level and arthritis index in rats induced arthritis. Such findings may offer DTX a promising anti-RA drug, and further studies on its effects may be needed.

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