

A Clinicopathological Study Comparing the Treatment Effect of Stem Cell Transplantation and Antivascular Endothelial Growth Factor (Bevacizumab) on Adjuvant Induced Rheumatoid Arthritis in Rats Joints

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Abstract: Background: Methotrexate is effective in rheumatoid arthritis treatment but with no regeneration of the damage tissue. Efforts to discover new target therapies are still needed. **AIM:** The present work studied the therapeutic and regenerative effect of stem cell transplantation as well as combined bevacizumab with methotrexate on the joints of animal model of rheumatoid arthritis compared to the standard methotrexate treatment by clinical, laboratory and histopathological examination. **Material and methods:** This study was carried out on 32 rats. Systemic arthritis was induced then clinical arthritis score was assessed. Anti-cyclic citrullinated peptides (CCP) level was measured. Two rats were sacrificed to determine their joint histopathological score. The remaining 30 rats were divided into three groups: **(A):**10 rats were injected with stem cells, **(B):**10 rats were injected with methotrexate, and **(C):**10 rats were injected with methotrexate and bevacizumab. After treatment, animals were clinically scored and measured for anti-CCP level. Then animals were sacrificed for histopathological analysis of their joints. **Results:** Improvement with the three modalities of treatment was found by clinical, laboratory and histopathological examination. Group (A) was the best regarding clinical and anti-CCP results while group (C) was the best regarding the histopathological vascular changes. **Conclusion:** Both bevacizumab and stem cells are promising therapies in the treatment of rheumatoid arthritis.

[Shaymaa Ali, Somaia Ahmed Saad El-Din, Hebt Alla El Chamy, Takwa Badr, Faten Ghazal, Hanaa Amer, Fatma A. Abu Zahra, Nadia Kamel and Ahmed Abdalla **A Clinicopathological Study Comparing the Treatment Effect of Stem Cell Transplantation and Antivascular Endothelial Growth Factor (Bevacizumab) on Adjuvant Induced Rheumatoid Arthritis in Rats Joints.** *Stem Cell* 2017;8(4):116-124]. ISSN: 1945-4570 (print); ISSN: 1945-4732 (online). <http://www.sciencepub.net/stem>. 20. doi:[10.7537/marscj080417.20](https://doi.org/10.7537/marscj080417.20).

Key words: Rheumatoid arthritis, methotrexate, mesenchymal stem cell, bevacizumab.

Abbreviation: Rheumatoid arthritis (RA), Anti-cyclic citrullinated peptides (Anti-CCP), human umbilical cord mesenchymal stem cell (HUC-MSC), Methotrexate (MTX), Enzyme linked immune sorbent assay (ELISA).

1. Introduction

Rheumatoid arthritis (RA) imparts a massive burden on health services worldwide. In spite of the big advances in the medical treatment of RA, uncontrolled active rheumatoid arthritis causes decreased quality of life and other comorbidities. Efforts to discover new target therapies have achieved considerable success (Liu *et al.*, 2010).

Several guidelines for management of rheumatoid arthritis exist. Disease-modifying antirheumatic drugs (DMARDs) and biologic agents slow disease progression and can induce disease remission in some patients. Methotrexate (MTX) is the most commonly prescribed DMARD (Burch and Onysko, 2012).

Mesenchymal stem cells (MSCs) have been largely studied and used as a new therapeutic tool for a

number of clinical applications, in particular for the treatment of rheumatologic disorders (Mumus *et al.*, 2011).

Bevacizumab (Avastin®) is a recombinant humanized monoclonal antibody against vascular endothelial growth factor and can inhibit angiogenesis that is now generally accepted to play a central role in maintaining and promoting RA (Kruse *et al.*, 2013).

This study is designed to assess the role of human umbilical cord mesenchymal stem cell transplantation and bevacizumab versus conventional methotrexate therapy on the joint of animal model (rats) after adjuvant induced arthritis using the Complete Freund's Adjuvant (CFA), followed by their clinical, laboratory (using the anti CCP antibodies) and histopathological assessment.

2. Material and Methods:

This study was carried out on 32 adult male albino rats of Wistar strain with approximate age and weight of 5 to 7 months and 200 to 250 gm respectively, housed in the Animal Facility of Medical Research Center, Faculty of Medicine, Ain Shams University with approval from ethics committee of Ain Shams University.

All rats were apparently healthy at the time of starting the experiment with no evident joint problem (no swollen joints, limping, deformities or aggressive behavior denoting pain). Systemic arthritis was induced in all rats by injection with 0.05 ml of Complete Freund's Adjuvant CFA (**Sigma, USA**) containing 1 mg/ml of heat-killed mycobacteria into one of the tail veins. One week later, they were subjected to a subcutaneous booster dose of 0.01 ml of the same material at the base of the tail (Lorentzen, 1999). Each rat was assessed clinically every 2 days and clinical arthritis was scored on a scale of 0-4, where 0 = no swelling, 1 = redness, 2 = swelling, 3 = digit deformity, and 4 = paw deformity (ankylosis) for each paw (Cremer, *et al.*,1990). Three weeks after induction of arthritis, blood samples were collected from all 32 rats in sterile tubes by insertion of capillary tubes into the retro-orbital plexus then they were measured for anti-CCP level using ELISA technique (Vanherok *et al.*,1998). For accurate calculation of the cut off value of anti-CCP level, a control group of 10 apparently healthy rats matched with our studied group of rats regarding weight and age was subjected to measurement of anti-CCP level.

Then, two rats have been sacrificed for histopathological assessment of their joints to ensure successful induction of arthritis and to determine their histopathological score.

Human Umbilical Cord Blood samples (hUCB) were obtained from the labor room of Obstetric and Gynecology Department, Faculty of Medicine, Ain Shams University, after obtaining an informed consent. By strict aseptic techniques, 50 ml of hUCB were withdrawn by milking from the umbilical vein and collected in sterile 15 ml Falcon tubes containing 2 milliliters of Acid Citrate Dextrose (ACD) anticoagulant (Eichler *et al.*,1999). Then stem cells were prepared at the laboratory of Medical Research Center, Faculty of Medicine, Ain Shams University using Bicol separating solution, centrifugation, aspiration using Fishing technique, washing by phosphate buffer saline and then the viability of isolated stem cells was determined and counted by a trypan blue exclusion test (Greish *et al.*,2012). **Then, the rats were divided into three groups; Group (A):** 10 animals were injected intraperitoneally with 1×10^6 mesenchymal stem cells (Greish *et al.*,2012). **Group (B):** 10 animals were injected subcutaneously with

methotrexate at a dose of 1mg/kg/week for 5 weeks according to (Morgan *et al.*,2001). **And Group (C):** 10 animals were injected with methotrexate at a dose of 1mg/kg/week for 5 weeks and two doses of intravenous avastin at a dose of 10mg/kg 2 weeks apart (Goff *et al.*,2009).

All animals were clinically scored according to the same previous score 5 weeks after the beginning of the treatment, followed by assessment of their anti-CCP level. Then, animals in all groups were sacrificed by intraperitoneal injection of 50 mg/kg thiopental sodium and they were subjected to the histopathological analysis of their synovium, articular cartilage and the subchondral bone (Dursun *et al.*,2009).

For preparation of histological sections:

The total knee joints were taken from skinned sacrificed animals and placed in 10% buffered phosphate formalin for 1 week before being subjected to acid decalcification in 3% nitric acid solution and maintained at room temperature for an average of 5-7 days. Decalcified joints were longitudinally sectioned, their synovium and cartilage were curetted and processed. Hematoxylin and eosin (H & E) stained sections were prepared with some sections were subjected to Masson's trichrome to assess subsynovial collagen fibres (Greish *et al.*,2012).

Assessment was done following a histological score for (Koizumi *et al.*,1999) and (Capitanescu *et al.*,2011).

Synovial analysis as follow: 1) Synovial lining cell hyperplasia: One or two layers, three or four layers, five or six layers and seven or more layers (0,1,2 and 3 respectively), **2) Inflammation in synovium:** no inflammation, perivascular aggregate, diffuse mild to moderate inflammation, dense diffuse inflammation, lymphoid follicle formation and germinal centers in the formed lymphoid follicles (0, 1, 2, 3, 4 and 5 respectively) **3) Extent of subsynovial collagen fibers deposition:** Absent, mild, moderate and sever (0, 1, 2 and 3 respectively) **4) Vascular changes:** Absent, vasodilation in subsynovial vessels, new small vessels at synoviocytes or extravasated RBCs, prominent subsynovial vasculature as granulation tissue, and endothelial proliferation with micro thrombosis (0, 1, 2, 3 and 4), **As regard the pannus formation:** Absent or present (0 or 1), **As regard articular cartilage destruction:** Absent, mild, moderate or severe (0, 1, 2 and 3 respectively), **As regard subchondral bone destruction:** Absent, mild, moderate or severe (0, 1, 2 and 3 respectively).

The collected data was revised, coded, tabulated and introduced to a PC using Statistical package for Social Science (**SPSS 15.0.1 for windows; SPSS Inc, Chicago, IL, 2001**). Data was presented and suitable analysis was done according to the type of data

obtained for each parameter, **descriptive statistics** include: Mean, Standard deviation (\pm SD), Standard error of mean (\pm SEM), range for numerical (quantitative) data and Frequency and percentage of non-numerical (qualitative) data.

Analytical statistics include: Student's "t" test, Mann Whitney test (U test), Chi-Square test, Paired "t" test and Wilcoxon Signed Ranks test with **P-value: level of significance as follow:** - $P > 0.05$: Non significant (NS), - $P < 0.05$: Significant (S), - $P < 0.001$: Highly significant (HS).

3. Results:

Clinical results:

Clinical arthritis was scored 3 weeks after induction of arthritis for all 32 rats and 5 weeks after starting of treatment for all 30 rats in the three groups on the previous described scale with maximal clinical score of 16 if all paws were fully ankylosed.

For group A, the clinical arthritis scores of animals was significant difference before and after treatment (decrease in the clinical score after treatment). Both group (B) and (C) showed no significant difference in the clinical score after treatment (table 1), with no significant difference in the clinical score between the three groups after treatment (table 2).

The clinical arthritis scores of the 2 rats that were sacrificed 3 weeks after induction of arthritis were 12 and 14.

Laboratory results (Anti- CCP):

Serum level of anti-CCP was measured 3 weeks after start of induction of arthritis (before start of treatment) for all 32 rats (cases) and remeasured again 5 weeks after start of treatment in all 30 rats of the three groups (group A, B and C) and also for 10 healthy rats as a control group. The anti-CCP levels ranged from 1.8-86.0mg/dl with a mean \pm SEM of 14.93 ± 3.17 for cases group (before treatment), while they ranged from 0.0-10.3mg/dl with a mean \pm SEM of 3.01 ± 1.03 for controls group with a high significant difference between the cases and control group.

For group A, the anti-CCP levels of animals were significantly different before and after treatment. However, for group B and C, no significant difference before and after treatment was found (table 3). The comparison between the three treated groups (A, B and C) showed no significant difference in anti-CCP level after treatment (table 4).

The histopathological results:

Histopathological analysis according to the previous score was done for 2 rats 3 weeks after induction of arthritis and for the whole 30 rats of the 3 groups at the end of our research. All specimens were stained with H & E and some of them were stained with Masson's trichrome.

Regarding the histopathological examination of the 2 rats that were killed 3 weeks after induction, they showed increased synovial lining cell layers (synovial hyperplasia) from 6 to more than 7 layers (grades 2 and 3) (figure 1A), perivascular inflammatory aggregates as well as diffuse inflammation that appeared dense in some areas (grades 1, 2 and 3) (figure 1B and 1C), subsynovial moderate to marked fibrosis that is further highlighted by Masson's trichrome stain (grades 2 and 3) (figure 1D), vasodilatation (grade 1) with increased number of subsynoviocyte small blood vessels and extravasated RBCs within the synovium (grade 2) as well as prominent new vessels (grade 3) (figure 1E), pannus formation (figure 1F) with moderate to severe destruction of the articular cartilage (grades 2 and 3) (figure 2A, and 2B) and mild erosion of subchondral bone (grade 1) (figure 2C).

As regard the histopathological findings after treatment in the three examined groups, all showed evidence of improvement with variable degrees even in the same group when compared to the two untreated rats as follow, As regard synovial cell hyperplasia was whether absent, or mild (grades 0 and 1 respectively) (figure, 8), group A showed 60% of the cases with one or two layers of synovial cell lining (grade 0), while 40% showed three or four layers (grade 1) compared to group B which showed 70% grade 0 and 30% grade 1 while in group C, 70% showed grade 0 and 30% showed grade 1. As regard inflammatory cell infiltrate was whether absent, perivascular or diffuse (grades 0, 1, and 2 respectively), for group A, 50% were (grade 0) and 50% were (grade 1) compared to group B and C which showed 60% grade 0, 30% grade 1 and 10% showed (grade 2). As regard subsynovial fibers deposition was whether absent, mild, (figure 8) (further highlighted by Masson's trichrome stain Figure, 3 A, B and C) moderate or severe (grades 0, 1, 2 and 3 respectively), For group A, all cases 100% were (grade 1) compared to group B which showed 80% (grade 1) and 20% (grade 3), and group C, with 10% were (grade 0), 70% were (grade 1) and 20% were (grade 3). As regard vascular changes were whether absent, dilated vessels, new subsynoviocytes small vessels with extravasated RBCs (grades 0, 1, and 2 respectively), in group A, 30% were (grade 0), 40% were (grade 1) and 30% were (grade 2). For group B, 60% showed grade 1 and 30% showed grade 2 compared to group C which showed 60% grade 0, 40% grade 1. As regard Pannus formation was whether absent (figure 3 D) or present (grade 0 or 1 respectively), in group A, 60% of cases were (grade 0), while 40% were (grade 1) compared to group B which showed 30% grade 0 and 70% grade 1, while group C showed 40% grade 0 and 60% grade 1. As regard articular cartilage destruction was whether

absent or, mildly (figure 3 E), or moderately destroyed (grades 0,1, and 2 respectively), in group A, all cases 100% were (grade 1), for group B 80% showed grade 1, and 20% showed grade 2 compared to group C which showed 20% grade 0, 70% grade 1 and 10% grade 2. Finally, As regard subchondral bone destruction was whether absent or mildly eroded (grades 0 and 1 respectively), For group A, all cases (100%) were (grade 1), for group B 10% showed grade 0, 80% grade 1 and 10% showed grade 2

compared to group C which showed 70% grade 0, and 30% grade 1.

Comparison between the three groups as regard the response to the treatment showed just significant vascular improvement in group (C) compared to group (B) with significant reduction in subchondral bone erosion in group (C) compared to both groups (A and B). Otherwise, no any significant difference between the three examined groups was found (tables 5 and 6).

Table (1): Clinical data for animals in the 3 groups:

Gr	No	Clinical score	Range	Mean±SD (±SEM)	Z	P	Sign
A	10	Before treatment	6-14	9.30±2.35 (±0.74)	2.81	<0.05	S
		After treatment	0-8	3.90±2.76 (±0.87)			
B	10	Before treatment	6-14	9.80±2.34 (±0.74)	0.76	>0.05	NS
		After treatment	0-12	5.20±3.76 (±1.16)			
C	10	Before treatment	8-14	9.60±2.01 (±0.63)	0.45	>0.05	NS
		After treatment	0-12	4.60±3.23 (±1.02)			

Z: level of Wilcoxon Signed Ranks test, P: level of significance, S: significant, NS: non significant

Table (2): Comparison between the groups A & B, A & C and B & C regarding the change in clinical score after treatment using Mann-Whitney U test:

Group	Mean±SEM	Z	P-value	Sign.
A	5.40±0.87	0.61	>0.05	NS
B	4.60±1.04			
A	5.40±0.87	0.03	>0.05	NS
C	5.00±0.84			
B	4.60±1.04	0.23	>0.05	NS
C	5.00±0.84			

Z: level of Mann-Whitney U test, P: level of significance, NS: non significant

Table (3): Anti-CCP levels for animals in the 3 group

Gr	No	Levels of anti-CCP	Range	Mean ±SEM	Z	P	Sign
A	10	Before treatment	2.5-15.7	8.15±1.26	2.09	<0.05	S
		After treatment	0.0-11.7	6.34±1.23			
B	10	Before treatment	1.8-86.0	15.86±8.00	1.18	>0.05	NS
		After treatment	1.8-32.3	10.23±3.38			
C	10	Before treatment	5.2-63.3	16.78±5.44	1.78	>0.05	NS
		After treatment	2.0-43.0	14.10±4.45			

Z: level of Wilcoxon Signed Ranks test, P: level of significance, S: significant, NS: non significant

Table (4): Comparison between the groups A & B, A & C and B & C as regard the change in anti-CCP level after treatment using Mann-Whitney U test:

Group	Mean ±SEM	Z	P-value	Sign
A	1.81±0.66	0.49	>0.05	NS
B	5.63±5.44			
A	1.81±0.66	0.98	>0.05	NS
C	2.68±2.79			
B	5.63±5.44	1.28	>0.05	NS
C	2.68±2.79			

Z: level of Wilcoxon Signed Ranks test, P: level of significance, NS: non significant

Table (5): Comparison between the 3 groups regarding the grade of vascular changes using Chi-square test:

Group	X ²	P- value	Sign.
A & B	3.50	> 0.05	NS
A & C	4.00	> 0.05	NS
B & C	10.40	< 0.05	S

X²: level of Chi-square test, P: level of significance, S: significant, NS: non significant

Table (6): Comparison between the 3 groups regarding the extent of subchondral bone affection using Chi-square test:

Group	X ²	P- value	Sign
A & B	2.22	> 0.05	NS
A & C	10.76	< 0.05	S
B & C	7.73	< 0.05	S

X²: level of Chi-square test, P: level of significance, S: significant, NS: non significant

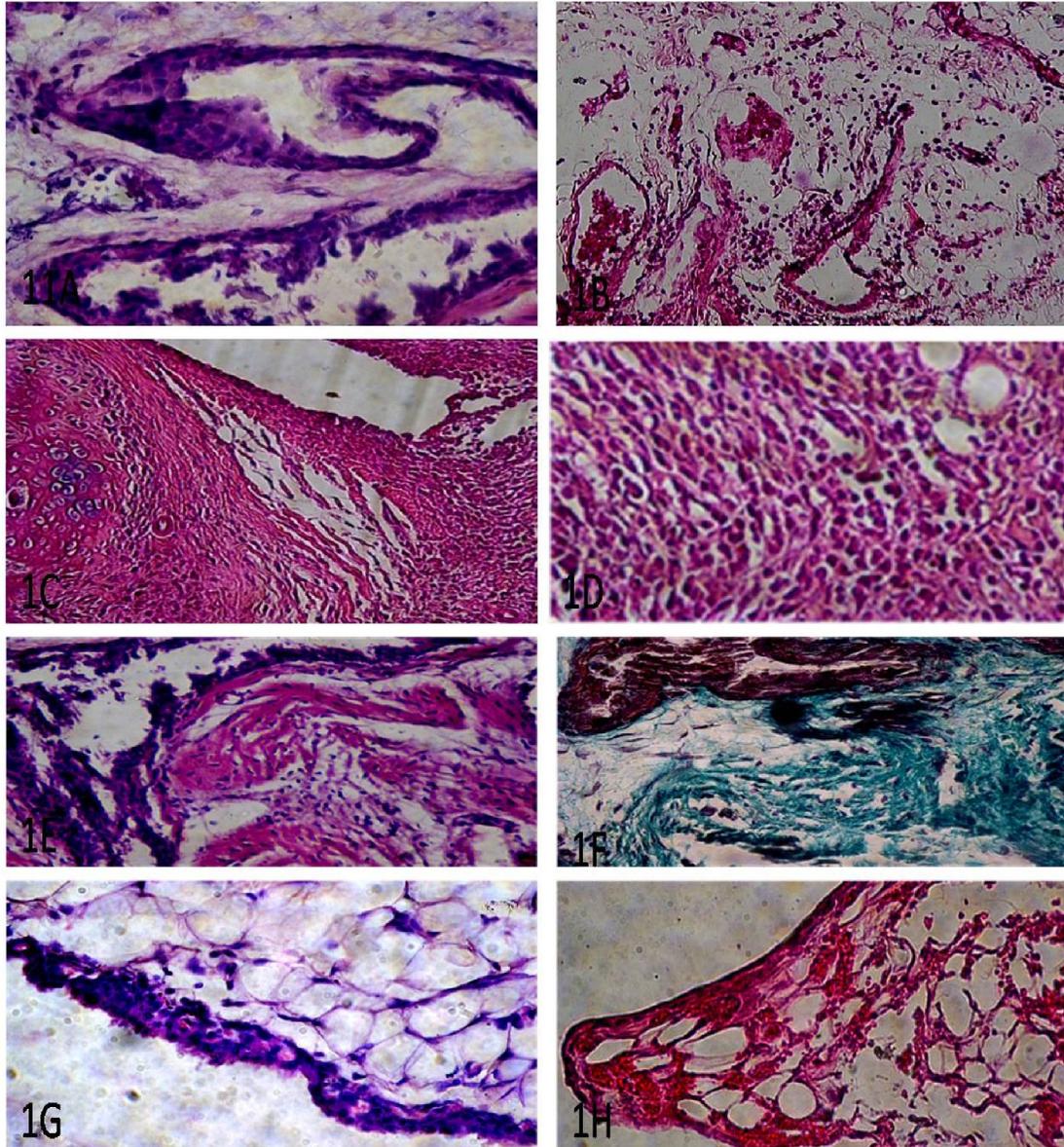


Figure (1) (A-H): Histopathological features of synovium before treatment: (A) Prominent synovial cell hyperplasia reaching 6 and 7 layers (grade 3) (H & E, x400) (B): Subsynovial inflammation with vasodilatation (grade 1) and perivascular inflammatory cellular aggregate (grade 1) (H & E, x100) (C) Subsynovial dense inflammatory reaction (grade 3) with pannus formation that moderately destroying the articular cartilage (grade 2) (H & E, x200), (D): higher power showed the dense inflammatory reaction (H & E, x400). (E) Subsynovial moderate fibers deposition (grade 2), (H & E x200), (F): Masson's trichrome stain showed the green color area of fibers deposition (collagen fibres) x400. (G): Small vessel formation at the synovial lining (grade 2) (H & E) x400. (H) Extravasated RBCs with prominent increase in the subsynovial blood vessels (grade 3) (H & E, x200)

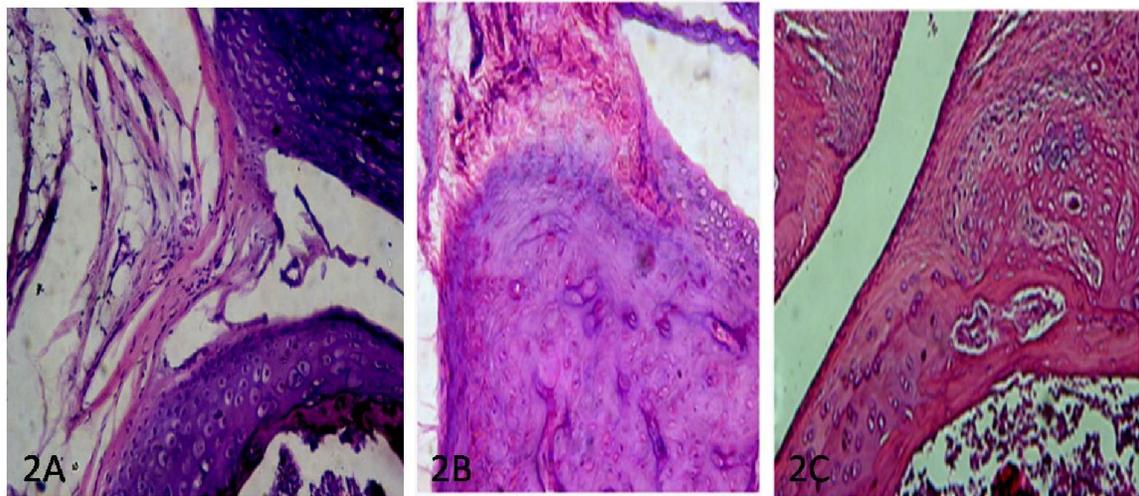


Figure (2) (A-C): Histopathological features of articular cartilage and subchondral bone before treatment: (A) Pannus creeping on the surface of the articular cartilage H & E, x 200 (B): Pannus mildly destroying the articular cartilage, H & E, x200. (C): Pannus moderately destroying the articular cartilage (grade 2) and mildly extending to the subchondral bone (grade 1), H & E, x100.

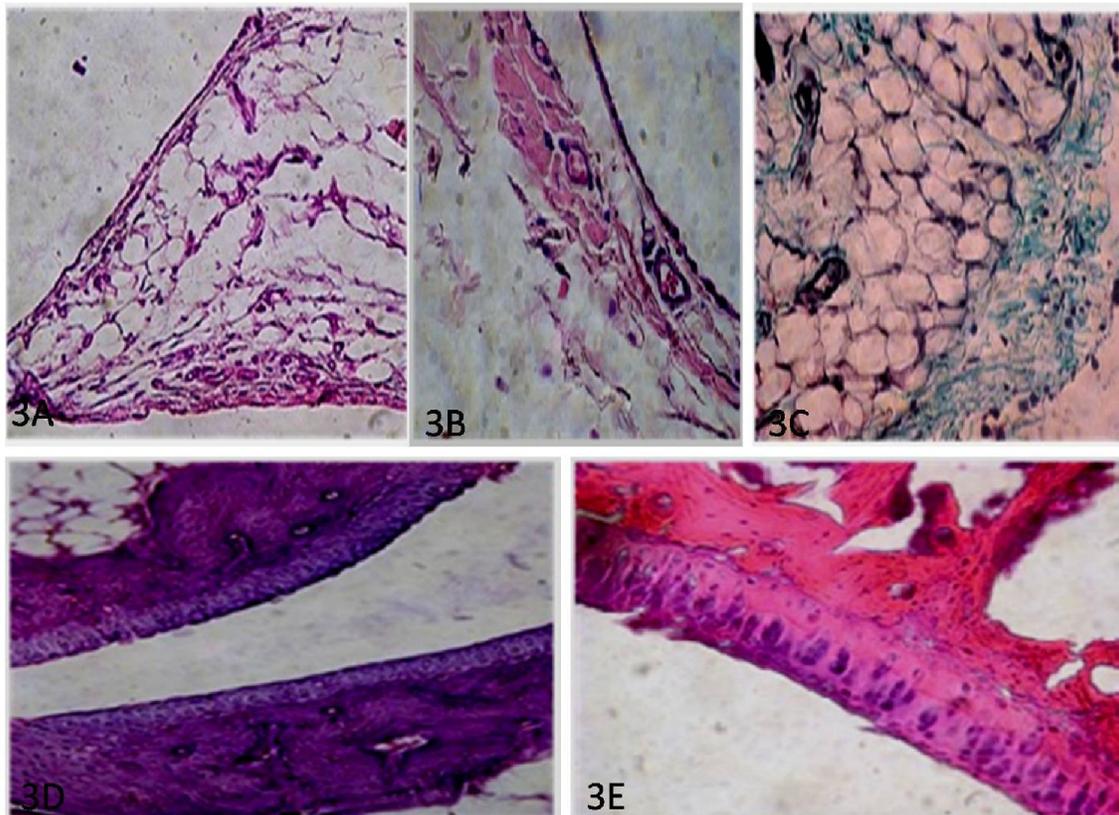


Figure (3) (A-E): Histopathological features of synovium, articular cartilage and subchondral bone after treatment (A): Single layer of synovial cell lining with no inflammation or vascular changes with minimal subsynovial fibers deposition, after treatment (H & E), x200, (B): Other case with similar findings at higher magnification x400, (C): Masson's trichrome stain showed decrease the green color area of fibers deposition compared to the diseased group x200. (D): Preserved articular cartilage and subchondral bone with no pannus formation, H & E x100, (E): Mild irregularity of the articular cartilage, H & E x200

4. Discussion

Although methotrexate can slow rheumatoid arthritis progression and can induce disease remission in most patients, it does not induce regeneration of the damaged tissues within the joints; especially the cartilage and it should be avoided before and during pregnancy (Burch and Onysko, 2012).

The present study was designed to study the therapeutic and regenerative effect of human umbilical cord mesenchymal stem cell (hUC MSCs) transplantation on the joints of animal model of RA compared to standard Methotrexate treatment. It was designed also to study the additional effect of Bevacizumab (Avastin®) to Methotrexate on joints by clinical, laboratory and histopathological examination, so systemic arthritis was induced in thirty two rats by injection of Complete Freund's Adjuvant CFA (Sigma, USA) containing heat-killed mycobacteria into one of the tail veins (Lorentzen, 1999).

The progression of the disease after three weeks was assessed using three parameters; clinical score, anti-CCP and histopathological examination of the joints (of two rats only), with induction of treatment in the remaining thirty rats which were divided into three groups, including group A (ten rats treated with stem cell), group B (ten rats treated with methotrexate) and group C (ten rats treated with both methotrexate with bevacizumab).

Clinically, all rats started to experience clinical signs of disease starting from day 10-12 after induction; these initial signs were restricted to redness in one or more paws which rapidly progressed to swelling, digit deformity and even to paw ankylosis in many rats by day 20. The clinical arthritis score ranged for all animals from 6-14 with a mean \pm SD of 9.80 ± 2.34 . This was in accordance with Seeuws *et al.*, 2010 and Griesh *et al.*, 2012 who induced arthritis in rats using the CFA and assessed the clinical arthritis with the same scoring system in this study during their work to assess the role of the MSC on arthritis, but they found 100% hind paw full ankylosis in immunized rats by the day 34 which was not found in our study as we started to treat the animals 3 weeks after immunization.

Regarding the serum level of anti-CCP antibodies, they were measured 3 weeks after induction of arthritis. They showed marked increase upon induction as 100% of immunized rats had detectable anti-CCP levels, while 90% of them were positive for the test showing levels ≥ 3.2 mg/dl (The cut off value in this study as previously mentioned). This is in accordance with Yu *et al.*, 2011 who measured the temporal kinetics of anti-CCP antibody during the course of adjuvant induced arthritis in rats

and found that the levels of anti-CCP paralleled that of the disease severity in untreated arthritic rats.

In this study, the histopathological examination of the joints of the two rats in whom induction was done without treatment showed prominent inflammatory changes and provide a scoring level. The joints showed successful induction of RA as evidence by synovial lining cell hyperplasia, diffuse inflammatory mononuclear and lymphocytic cell infiltration, vasodilatation with increased number of subsynoviocyte blood vessels and extravasation of RBCs, moderate-to-severe subsynovial fibrosis with pannus formation that markedly invading and destructing the articular cartilage with mild erosion of the subchondral bone.

These findings are in accordance with Liu *et al.*, 2010 and Griesh *et al.*, 2012 who assessed the disease progress in the immunized group of rats and showed that the first histological signs of disease appeared at day 10, when hypertrophy of the synovial lining was observed. Massive mononuclear cell infiltration of the synovium was also visible in the same section with evidence of hypervascularity and congestion. With disease progression, by day 21 the synovial tissue became hypercellular and well vascularized, so that, at this stage, the synovial lining had been replaced by an inflammatory granulation tissue, or pannus obliterating the joint space. By day 34, infiltrating pannus significantly eroded the articular cartilage and subchondral bone resulting in complete fibrous ankylosis.

Examination of sections stained with Masson's trichrome showed increased green color area with increased fibers deposition which confirmed the findings found on H & E sections. This is in accordance with Griesh *et al.*, 2012 who found that there was increase in green color area percentage with image analyzer examination in positive control group which was injected with CFA with no treatment.

Treatment of animals in this study started 3 weeks after induction of arthritis and continued for 5 weeks. The animals were divided in three groups; group A treated with mesenchymal stem cells (MSCs), group B treated with Methotrexate (MTX) while group C treated with MTX with Bevacizumab.

Regarding the clinical score, all the three treated groups showed reduction in the clinical score after treatment with the best response in the MSC group as 100% of rats showed decline in the clinical score after treatment with a statistical significant difference between the clinical score after treatment and before treatment. Only 80% of rats in the MTX group and 90% of them in MTX+Bevacizumab group showed decline in the clinical score after treatment with no statistical significant difference between the score

after treatment and before treatment. This is in accordance with Mao *et al.*, 2010 and Griesh *et al.*, 2012 who showed that the decrease in the clinical score was more in MSCs group than other groups. These findings are going more with the immunomodulatory effect of MSCs (Waterman RS *et al.*, 2010).

Regarding the levels of anti-CCP, all the three treated groups showed reduction in the anti-CCP levels after treatment with the best response in the MSC group and MTX+Bevacizumab group as 80% of rats showed decline in the levels of anti-CCP after treatment with a statistical significant difference between the anti-CCP levels after treatment and before treatment in MSC group. Only 60% of rats in the MTX group showed decline in the anti-CCP levels after treatment with no statistical significant difference between the levels after treatment and before treatment.

This is in accordance with Yu *et al.*, 2011 who measured the temporal kinetics of anti-CCP antibody during the course of AIA in rats. They found that the treated rats with attenuated disease had reduced anti-CCP levels, while rats with aggravated disease had increased levels of anti-CCP.

Analysis of histological sections stained with H & E of rats 5 weeks after treatment showed reduction in the inflammatory signs in all groups in comparison to the untreated rats. Regarding synovial lining cell hyperplasia, inflammatory cell infiltration in the synovium, subsynovial fibrosis, vascular changes, pannus formation and the extent of the articular cartilage destruction and subchondral bone erosion, the grades of each decreased in comparison to the untreated rats with significant improvement in the vascular changes and the subchondral bone erosion in group (C) (treated with both mesotrexate and bevacizumab).

Griesh *et al.*, 2012 showed that there was a significant reduction of hyperplasia of the synovial tissue with mild obliteration of the joint cavity in MSC treated rats in comparison to the MTX group. These findings were similar to Liu *et al.*, 2010 who observed that control mice exhibited a marked mononuclear cell infiltration, severe synovitis, pannus formation and bone erosion, while the majority of joints from mice injected with MSCs had normal morphology with a smooth articulation cartilage surface and an absence of inflammatory cell infiltrate and pannus formation.

Our findings were confirmed by examination of the Masson's trichrome stained sections which showed decreased green color area with decreased fibers deposition within the synovium in response to treatment which was in agreement with Griesh *et al.*, 2012 who found that there was decrease in green color

area percentage in MSCs and MTX groups in comparison to the untreated group.

In conclusion, this study showed great response to all treatment strategies compared to untreated animals, with the mesenchymal stem cells had given the best effect on the clinical score and anti-CCP level. Moreover, this study showed that addition of Bevacizumab to standard Methotrexate treatment can decrease the vascular changes and neoangiogenesis. However, further studies on larger number of rats for longer period to detect the possible side effects of MSCs and Bevacizumab and permission of further studies in humans after full researches on animals are needed.

Conflicts of interest:

There are no conflicts of interest.

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10/29/2017