

A Clinicopathological Study Comparing the Therapeutic Role of the Mesenchymal Stem Cell and Bevacizumab as Anti Vascular Endothelial Growth Factor on the Lung as an Extra-articular Target in Adjuvant Induced Arthritis in Rats

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Abstract: Background: Rheumatoid arthritis (RA) is a chronic systemic inflammatory disease, characterized by polyarthritis and extra-articular organ disease. Lung disease occurs commonly in rheumatoid arthritis and associated with significant morbidity. It is the second leading cause of RA-associated mortality. **Aim:** Assess the effect of Stem cell transplantation and anti-vascular endothelial growth factor (Bevacizumab/avastin) on rheumatoid arthritis extra-articular organs (concerning the lung as extra articular target for the disease) in adjuvant induced arthritis in rats compared to conventional mesotrexate therapy. **Material and Methods:** This study was carried out on 34 adult male albino rats. Systemic arthritis was induced in all rats, each rat was assessed clinically and scored. Three weeks after induction of arthritis, blood samples were collected and measured for anti cyclic citrullinated peptides (anti-CCP) level using ELISA technique, then two rats had been sacrificed to assess the histopathological score of their organs (include lung, liver, kidney, spleen and heart) and it was found that lung was the only organ showed significant histopathological features, thus it was the only assessed organ in the treated groups follow up. Thirty rats were divided into three groups as follows: **Group (A):**10 animals were injected with mesenchymal stem cells, **Group (B):** 10 animals were injected with methotrexate, **Group (C):**10 animals were injected with methotrexate and avastin. Two rats kept without treatment as a control. Five weeks after the beginning of the treatment, all animals were clinically scored, followed by measuring anti-CCP level, then animals in all groups were sacrificed, followed by histopathological analysis of their lungs. Statistical analysis of the clinical, laboratory and histopathological findings was done. **Results:** The lung was the only organ showed significant histopathological affection. There was improvement in all treated groups. The results of Bevacizumab combined with Methotrexate treated group were close to stem cell treated group while the least improvement was in MTX treated group. **Conclusion:** The lung is common target in RA. Both mesenchymal stem cell and bevacizumab are promising therapies to improve the extra articular lung manifestations of RA, but more studies are needed on larger number of rats and for longer duration to assess the possible side effects of MSCs and bevacizumab.

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Key words: Rheumatoid arthritis, mesenchymal stem cell, bevacizumab, cyclic citrullinated peptide, histopathology

Abbreviations: Rheumatoid arthritis (RA), human umbilical cord mesenchymal stem cells (HUC- MSCs), anti-vascular endothelial growth factor (anti VEGF), cyclic citrullinated peptide (CCP), mesotrexate (MTX)

1. Introduction

Rheumatoid arthritis is a chronic inflammatory disease diagnosed on clinical assessment of the affected joints and some blood tests include rheumatoid factor and more recently anti citrullinated peptide antibodies. Then it can be supported by the presence of characteristic histopathological findings in the affected organs that can provide simple grading

system to study the pathogenesis and the effects of new therapeutic strategies (Ellen et al, 2014). It is associated with a range of extra-articular features which manifest in several body systems. They can seriously affect the cardiovascular and cardiopulmonary systems and usually associated with increased morbidity and mortality (Paloma, 2014). The pulmonary complications ranked as the second

major cause of death in this patient population (Young et al., 2007). Lung involvement can be in the form of parenchymal nodules, interstitial involvement, and chronic obstructive airway disease. Rheumatoid pulmonary vasculitis is rare (Schneider et al., 2012).

The associated lung disease may be due to, rheumatoid associated lung disease, infection secondary to immunosuppression, or drug induced. There are several drugs of proven articular efficacy to be associated with accelerated respiratory failure in the presence of interstitial lung disease. The most frequently implicated RA drug leading to pulmonary toxicity is methotrexate (MTX), with 20% increased mortality rate, especially if the drug toxicity occurred in the first six months of treatment. More target therapies are in need (Malik et al., 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. MSCs indeed have therapeutic potential for bone and joint diseases due to their multipotent differentiation abilities and the secretion of a variety of cytokines and growth factors that confer on their anti-fibrotic, anti-apoptotic, pro-angiogenic and immunosuppressive properties (Maumus et al., 2011).

Moreover, neoangiogenesis is recognized as a key event in the formation and maintenance of inflammation in rheumatoid arthritis, so targeting blood vessels in RA may be an effective future therapeutic strategy. Vascular endothelial growth factor (VEGF) has been demonstrated to have a central involvement in the angiogenic process in RA. Bevacizumab is a "monoclonal antibody" that works by interfering with the process of angiogenesis by targeting and inhibiting human vascular endothelial growth factor. Anti-angiogenic drugs such as bevacizumab may play a significant role in longstanding RA (Malik et al., 2012).

This work was designed to study the therapeutic and regenerative effect of human umbilical cord mesenchymal stem cells ((hUC-MSCs) as well as bevacizumab combined with methotrexate on the affected extra articular tissue (concerning the lung as common disease target) in the animal model of RA versus the standard MTX therapy.

2. Material and Methods

This study was carried out on 34 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.

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. Twenty days (3 weeks) after induction of arthritis, blood samples were collected from all 34 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 (*Van Herck 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. Using the data of anti-CCP level for cases and controls groups and by using the ROC curve (Reciprocal Operator Characteristics), we selected the best cut off value for anti-CCP in our samples which was 3.2 mg/dl as this gives the best sensitivity (90%) and specificity (80%).

After blood samples collection, two rats have been sacrificed and their joints were examined histopathologically to ensure successful induction of arthritis as well as their lung, liver, spleen, kidney and heart with determination of their histopathological score (the lung was the only organ showed significant histopathological findings so it was our target to assess the treatment response in the treated groups).

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 thirty two rats were divided into three groups as follows: Two rats left without treatment as control: **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*). **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 thirty four (34) days 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 lungs (**Dursun et al., 2009**).

Preparation of histological sections

Lung dissection was done (as well as the liver, the kidney, the spleen and the heart). For lung, a fixative solution (10% neutral-buffered formalin) was perfused through its main bronchus. The lung (as well as the other examined organs) was immersed in the fixative for 24 – 48hr, followed by tissue processing as follows: dehydrated in a graded series of ethanol, clearing by xylene, impregnation in melted paraffin then embedding with paraffin blocks preparation, cut into 5-mm-thick serial sections and stained with hematoxylin – eosin and Masson's trichrome to highlight the fibrosis (collagen fibers).

The severity of pulmonary affection was done on a numerical scale in a blind evaluation by two pathologists. According to the findings, different scales with some modifications were applied on our

examined sections including (**Oyarzun et al., 2005**) as follows: Thelymphoplasmacytic infiltration, the lymphoid follicle formation, the epithelioid granulomas formation, the neutrophilic infiltration, the congestion, and the hemorrhage. Each reported parameter was assessed according to the severity into absent, mild, moderate or severe with 0, 1, 2 and 3 scores respectively. Also assessment of vasculitis whether absent (0) or present (1) was done with total score for all these parameters of 19. All these pathological findings were further examined for the extent of lung affection, whether minimal, mild, moderate, severe or extensive with 1, 2, 3, 4 and 5, respectively. Then, total score of 24 for lung inflammation was applied with 1to8 considered mild lung inflammation, 9to16 considered moderate lung inflammation and 17to24 considered severe lung inflammation. Further assessment for the emphysema whether absent, mild, moderate or severe with 0, 1, 2 and 3 scores and vessel wall hyperplasia and hypertrophy was scored whether absent (0) or present (1).

Then, to assess the lung fibrosis, each section was examined by the light microscope with average 10 examined fields by ten objective lens then mean score is taken according to **ASHCROFT et al., (1988)**. Masson's trichrome stain was applied for further illustration of fibrosis (**table 1**).

Table 1: ASHCROFT scale (ASHCROFT et al., 1988).

Grade of fibrosis	Histological features
0	Normal lung
1	Minimal fibrous thickening of alveolar or bronchiolar Walls
2	
3	Moderate thickening of walls without obvious damage to lung architecture
4	
5	Increased fibrosis with definite damage to lung structure and formation of fibrous bands or small fibrous Masses
6	
7	Severe distortion of structure and large fibrous areas; "honeycomb lung" is placed in this category
8	Total fibrous obliteration of the field

If there was overlap between two odd numbers, the even number in between was used.

Finally total lung score of 36 was applied that represent summation for all histopathological findings with 1to9 represent mild lung affection, 10to18 represent moderate lung affection, 19to27 for severe lung affection and 28to36 for profound lung affection.

Statistical analysis of data: The data for all parameters were expressed as mean \pm S.D. The treated groups were compared before & after treatment and for significance calculation paired student *t*-test was used with $p < 0.05$ (significant). Anti-CCP titre was

measured in 10 normal rats & the cut off value was calculated by mean + 2 S.D.

3. Results

As regards the clinical score: Clinical score after treatment induction was improved (decreased) in all treated groups with the best improvement in bevacizumab combined with MTX treated group with 6.7 difference, then MSCs treated group with 6.5 difference then MTX only treated group with 5.2

differences. Comparison between the clinical score for each group before and after treatment is shown in (table 2). Analysis of these findings using paired t-test

showed that P value was $>.05$, so there was significant improvement in the clinical score in the three groups before and after treatment (table 3 and 4).

Table 2: comparison between the clinical score, Anti-CCP level & total lung score before and after treatment of the three groups (including the untreated four rats)

Group	NO	Before treatment			After treatment		
		Clinical score	Anticcp level		Clinical score	Anticcp level	Histopathology
Stem cells group(A)	1	8	9.4		4	8.8	14
	2	8	5.9		0	1.1	5
	3	9	7		6	7.7	15
	4	10	8.8		3	8.7	11
	5	11	12.8		2	8.1	20
	6	11	15.7		3	11.7	20
	7	8	2.5		0	0	12
	8	10	9.5		2	8.8	18
	9	8	4		0	2	10
	10	8	5.9		6	6.5	6
MTX group(B)	11	8	1.8		0	2.5	9
	12	10	9.8		6	8.4	17
	13	12	20		10	27.5	13
	14	9	7.9		0	5.4	19
	15	12	86		6	32.3	16
	16	8	7.5		3	7	9
	17	11	15		6	19	13
	18	10	3.4		4	3.5	14
	19	9	2.5		4	3.8	10
	20	8	4.7		6	5	13
Combined group(C)	21	8	5.2		0	2	6
	22	12	63.3		6	43	20
	23	10	16.5		4	11.2	17
	24	8	7.9		4	5.6	18
	25	9	6.9		0	4.3	17
	26	9	7.2		6	7.4	6
	27	10	19.3		6	36	19
	28	10	7.7		0	4.6	11
	29	11	14.1		2	10.6	19
	30	12	19.7		4	16.3	16
	NO	3Wks after ind.			9Wks after ind.		
		Clinical score	Anticcp level	Histopathology	Clinical score	Anticcp level	Histopathology
Untreated rats	31	12	27.5		14	42.4	27
	32	10	3.7		13	18.2	18
	33	12	27.5	20			
	34	12	20	17			

Table 3: Statistical analysis of the groups regarding the clinical score and anti-CCP level before and after treatment

Parameter	Groups	Before TTTmean ±S.D.	After TTTmean ±S.D.	T- Value	P-Value	Significance
Clinical score	Stem cells group(A)	9.1±1.29	2.6±2.27	7.88	1.80962E-05	(S.)
	MTX group(B)	9.7±1.57	4.5±3.03	4.82	4.53847E-05	(S.)
	Combined group(c)	9.9±1.45	3.2±2.53	7.27	1.18895E-05	(S.)
Anticcp level	Stem cells group(A)	8.15±3.99	6.34±3.91	1.02	0.02424	(S.)
	MTX group(B)	15.86±25.31	11.44±10.84	0.51	0.44623	(N.S.)
	Combined group(c)	16.78±17.23	14.1±14.10	0.38	0.36265	(N.S.)

S. = Significant N. S.= not Significant

As regards the laboratory findings (anti-CCP): On day 20 after induction (before treatment) anti-CCP titre was detected in 100% of rats and positive (above the cutoff value) in 80% of rats. After treatment, titers of anti-CCP decreased in all treated groups that was only significant in group A with *P* value <.05 with group A while it wasn't significantly in groups B and C. Comparison between anti-CCP titre for each group before and after treatment is shown in (table 2). Analysis of these findings using paired t-test to assess the *P* is shown (table 3 and 4).

As regards the histopathological findings: The two rats, which were sacrificed after induction (On day 20 after induction) (without treatment) showed features of rheumatoid lung that included: dense lymphoid infiltration (Fig. 1a), bronchial wall fibrosis highlighted by Masson's trichrome stain (Fig. 1b), lymphoid follicle formation and epithelioid granulomas (Fig. 1c, 1d), neutrophilic infiltration, vasculitis (Fig. 2a, and 2b), alveolar septal congestion (Fig. 2c,2d), vessel wall hyperplasia and hypertrophy

(Fig. 3a), emphysema (Fig. 3b) and alveolar wall fibrosis (Fig. 3c) that was further highlighted by Masson's trichrome stain (Fig. 3d). Moreover, the two rats that were sacrificed 9 weeks after induction without any treatment showed prominent fibrosis with distorted lung architectural with more dense lymphoid infiltration, lymphoid follicle formation (Fig. 4a, 4b), multiple epithelioid granulomas, vasculitis with end arteritis obliterans and foci of dense neutrophilic infiltration (suppuration) (Fig. 4c,4d).

In the treated groups, there was an obvious improvement & decrease of infiltration by inflammatory cells, lymphoid infiltration & granulomas with restoration of the lung architecture and less fibrosis (Fig. 5a,5b,5c,5d) in all treated groups with Bevacizumab combined with MTX group & Stem cells group showed the best histopathological improvement as 20% of the cases in each of them were equally improved (the least score). The improvement was variable between the groups and even within the same group (tables 2).

Table 4: The Difference in the groups before and after treatment and the Percentage of improvement regarding both clinical score and anti-CCP level

Parameter	Group	difference mean	No. of improved rats (no%)
Clinical score	Stem cells group (A)	6.5	10 (100%)
	MTX group (B)	5.2	10 (100%)
	Combined group (C)	6.7	10 (100%)
Anticcp level	Stem cells group (A)	1.81	8 (80%)
	MTX group (B)	4.42	4 (40%)
	Combined group (C)	2.68	8 (80%)

Table 5: The mean and the SD of all histopathological parameters in all treated groups:

Group	severity								total inflammatory score	fibrosis	emphysema	vessel wall (Hyperplasia/Hypertrophy)	total lung score
	chronic inflammatory cells	lymphoid follicle formation	epithelioid granulomas	neutrophilic infiltration	congestion of alveolar septa	hemorrhage	vasculitis	extent of inflam.					
Stem cells group(A)	1.8±0.63	0.9±0.88	0.9±0.74	1.1±0.57	1.4±0.7	0.9±0.57	0.2±0.42	1.9±0.88	9.1±3.81	1.8±0.92	1.8±0.63	0.4±0.52	13.1±5.32
MTX group(B)	1.5±0.53	0.5±0.85	0.8±0.63	1.1±0.32	1.8±0.79	1.5±0.53	0.6±0.52	1.4±0.7	9.2±2.53	1.8±0.79	1.9±0.57	0.4±0.52	13.3±3.37
Combined group(c)	2.3±0.95	0.7±0.67	0.8±1.03	1±0.67	1.4±.52	1.3±0.48	0.3±0.48	2.2±0.92	10±3.53	2.6±1.35	1.9±0.74	0.4±0.52	14.9±5.3

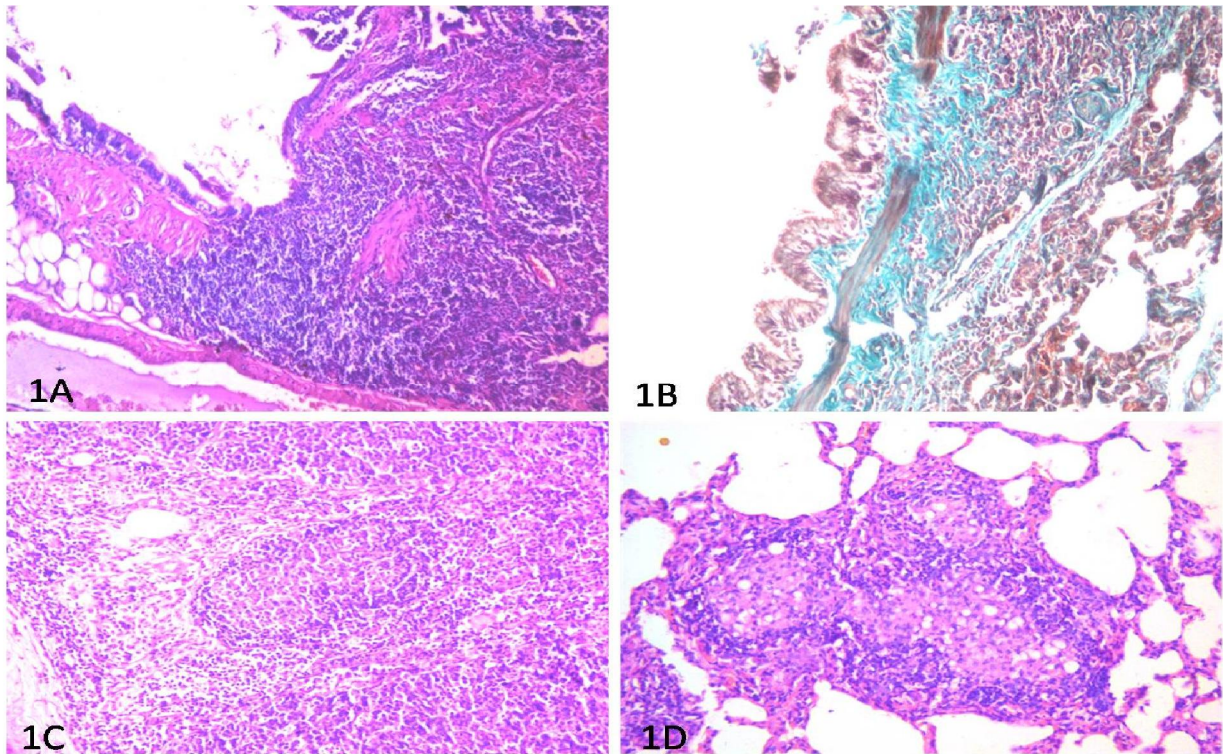


Figure 1 (a, b, c, d): 1a) Dense lymphoid infiltration destroying the muscle layer of the bronchiole H & E x100, **1b)** Masson's trichrome stain highlights the greenish fibrous tissue that disrupt the muscular coat of the bronchiole x200, **1c)** Lymphoid follicle formation in the lung tissue H & E, x200, **1d)** Many epithelioid granulomas within the lung tissue H & E, x200.

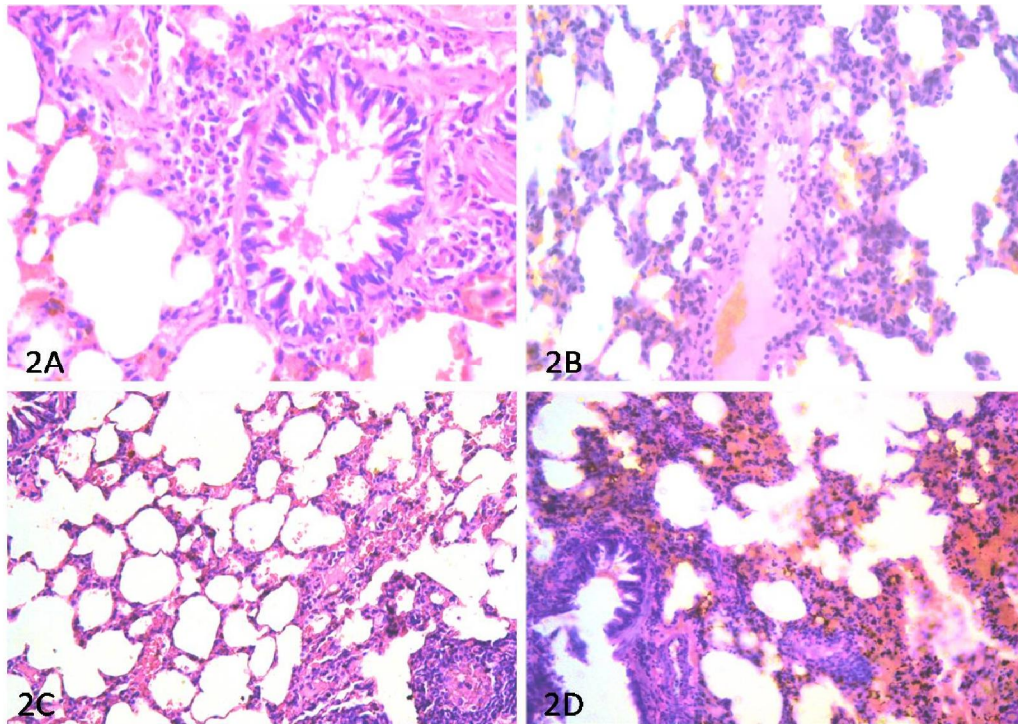


Figure 2 (a, b, c, d): 2a) Peribronchiolar mixed inflammatory cells (with prominent neutrophils), H & E, x400, **2b)** Vasculitis as evident by infiltration of the vessel wall by inflammatory cells H & E, x200, **2c)** Congested alveolar septa H & E, x 100, **2d)** Prominent hemosiderosis in the lung tissue H & E x200.

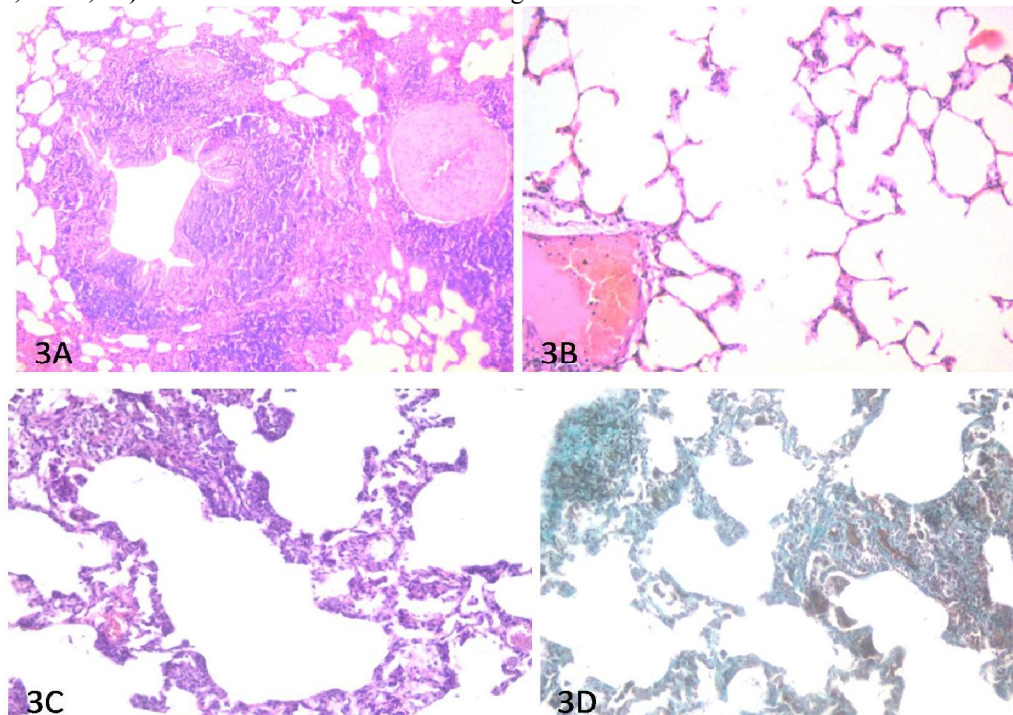


Figure 3 (a, b, c, d): 3a) Vessel wall hyperplasia and hypertrophy presents nearby inflamed bronchiole H & E, 100, **3b)** Prominent disrupted alveolar septa (emphysema) H & E, x200, **3c)** Moderate to marked thickened alveolar septa with some are disrupted associated with distorted lung architectural, Aschroft fibrosis score 5, H & E, x200, **3d)** Masson's trichrome stain highlight the greenish fibrous tissue, x200.

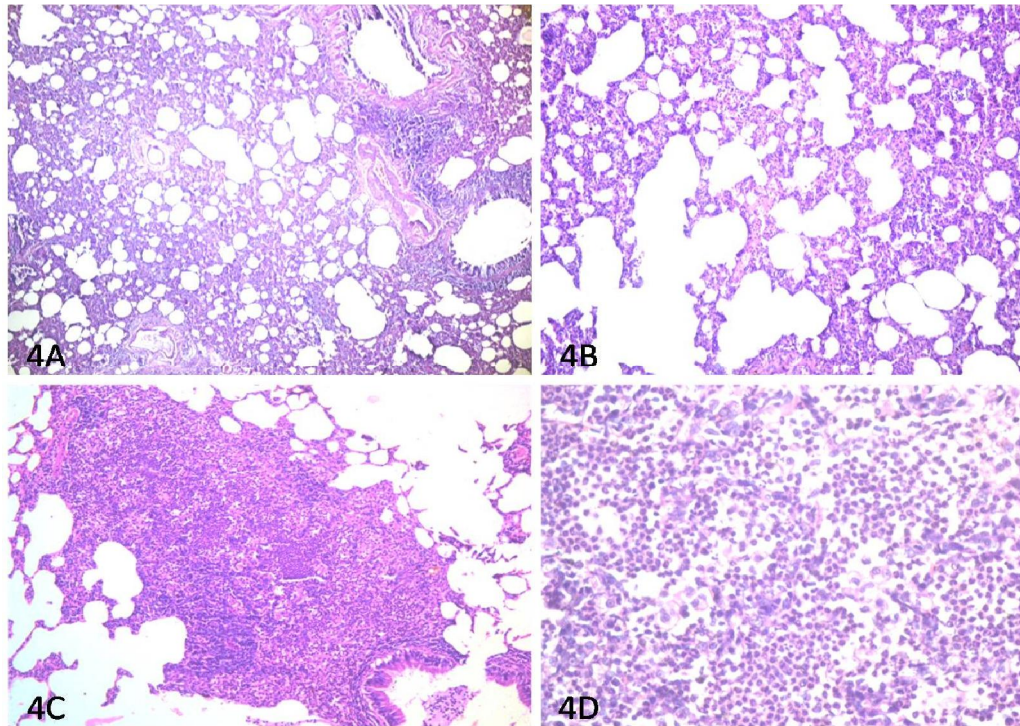


Figure 4 (a, b, c, d): 4a) Dense lung inflammation with distorted lung architectural H & E, x40, 4b) Higher magnification H & E x100, 4c) Focus of dense lung inflammation showed neutrophils, pus cells and chronic inflammatory cells (micro abscess) H & E, x 100, 4d) Higher magnification, x400.

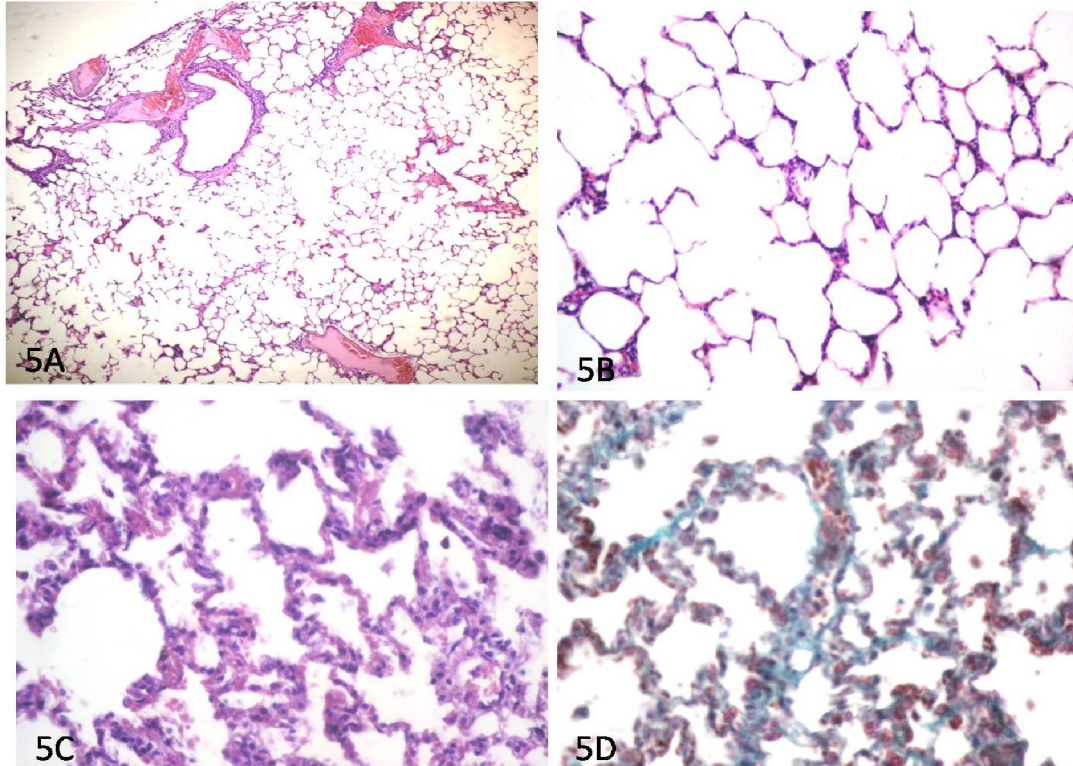


Figure 5 (a, b, c, d): 5a) Marked reduction in the inflammation after treatment H & E, x40, 5b) higher magnification H & E, x200, 5c) Mild to moderate thickened alveolar septa showed fibrosis grade 2 (according to Ashcroft scale) H & E, x200, 5d) Masson's Trichrome stain highlights the greenish fibrous tissue, x200.

4. Discussion

Rheumatoid arthritis (RA) is the most common inflammatory joint disease, affecting 1% of the population worldwide. It is a systemic inflammatory disease that involves articular as well as extra-articular structures. Rheumatoid arthritis is associated with a high risk of morbidity and mortality secondary to the early development of cardiovascular, lung diseases and malignancy. Pulmonary involvement in RA is accounting for around 10-20% of the mortality associated with RA (**Rojas-serrano et al., 2012**).

This work was designed to study the therapeutic and regenerative effect of human umbilical cord mesenchymal stem cells (hUC-MSCs) as well as combined bevacizumab and mesotrexate on the extra articular manifestations of RA concerning the lung as a common target for the disease in the animal model of RA versus the standard MTX weekly therapy using three parameters for assessment that were the clinical, laboratory and histopathological parameters.

As regards the clinical score, after induction without treatment, it ranged from 8 to 12. These results were in accordance with **Greish et al., (2012) & Rajian et al., (2013)**, who found that 20 days after adjuvant injection, a steep increase in clinical score was observed with more than 90% of the rats developed arthritis & also according to **Lionel Philippe (1997)** who found that Rats were affected in all four paws, with the arthritic index increased until the end of the experiment (total score 9.8 ± 0.8 on *day 18*). This is similar to the present results that found a total score of (9.57 ± 1.43) in the whole 30 rats.

On 34th day after treatment all treated groups showed significant improvement of the clinical score with the best improvement was in group C (combined bevacizumab and mesotrexate treated group) and the least improvement was in group B (mesotrexate only treated group). The comparison between the clinical score before and after treatment in all treated groups showed statistical significant improvement.

Clinical score of group A (stem cells group) showed improvement better than that of MTX only treated group. Paw swelling had completely disappeared 21-34 days after stem cell administration. These results were in accordance with those of **Mao et al., (2010)** & as reported by **Greish et al., (2012)** that the overall arthritis signs totally disappeared at day 34 post treatment in MSC group, with a significant decrease in MSC treated group compared to MTX only treated group.

As regards anti-CCP titre, it was assessed before treatment to follow disease activity & effect of treatment on the rats & it was detected in 100% of rats. This was similar to the results of **Hua Yu et al., (2011)** who pointed out that negative anti-CCP antibody test does not exclude disease, but its high

specificity means that a positive result markedly increases the probability that the patient will have RA. High anti-CCP antibodies also identify a subset of patients who are likely to have substantial ongoing disease activity, accrue more damage, and who will probably benefit most from early aggressive treatment.

After treatment, anti-CCP titre decreased in all treated groups with group A (MSC treated group) and C (combined methotrexate and avastin treated group) showed the best improvement as 80% of the rats showed decreased anti-CCP level compared to 40% of the rats in group B. Comparison between anti-CCP level before and after treatment in the three examined groups showed significant improvement in group A only. This was in concordance with **Hua Yu et al., 2011** who found that anti-CCP is more likely to decrease in rats showing a greater degree of clinical improvement.

As regards the histopathological findings in the two rats that were sacrificed three weeks after induction and the other two rats that were sacrificed nine weeks after induction without treatment, successful induction were done **Schneider et al., 2012** reported that although most of the pathologic manifestations of RA seem to be nonspecific, two primary features emerge on review of many well-documented cases of RA-associated interstitial lung disease. First, there is often a prominence of lymphoid aggregates and follicles with germinal centers throughout the lung biopsy. Second, many RA lung biopsies, more than with any other rheumatic diseases, show concurrent acute, subacute, and chronic histological changes, should always raise a strong consideration of RA lung disease. These were in concordance with our results as the examined lung tissue showed prominent lymphoid infiltration with lymphoid follicles formation as well as foci of mixed inflammatory cells.

The three groups after treatment showed reduction in the histopathological findings (improvement) that present in the examined lung sections, but with variable degrees between the treated groups and even in the same group.

For group A, it showed the least score (best improvement) in the hemorrhage, vasculitis and emphysema with reduction in the inflammatory cells but less than group B, this was In contrast to **Liu, et al., 2010** who found on his work on rats, that the majority of joints from mice injected with UC-MSCs had normal morphology with a smooth articulation cartilage surface, and an absence of inflammatory cells infiltrate and pannus formation. Also **Greish et al., 2012** who found that stem cells treated group & MTX group were close to each other in leukocytic infiltration in rats joints. Till now, the experience of MSCs in the treatment of RA is limited to a few cases,

with controversial results from preclinical models (**Gonzalez-Rey et al., 2010**). Moreover, this study found that there was a significant positive correlation between anti-CCP level & lymphoid follicle formation and this was concordance with **Oosterhout et al., 2008** who found that lymphocyte infiltration is associated with anti-CCP-positive disease.

For group B, it showed the least inflammatory response with a reduction in the lymphoid infiltration, lymphoid follicle formation and the extent of lung inflammation. This was in concordance with **Greish et al., 2012** who found a significant reduction in the inflammatory cells in the joints of adjuvant induced arthritis with methotrexate therapy.

For group C, it showed the least score in the neutrophilic infiltration (best improvement), however, all groups showed nearly equal better reduction in vessel wall hyperplasia and hypertrophy.

Moreover, there were significant positive correlation between Anti-CCP level & lung fibrosis similar to that shown by **Alsahy et al., 2009** who reported that blood anti-CCP levels showed highly significant elevations in patients with affected lung than in patients without lung affection. The significant negative correlation they found in their work between anti-CCP and forced expiratory volume (FEV) % means that deterioration in lung function increases as anti-CCP increases. These findings were also reported by **Tureson et al., 2013**.

In conclusion, the lung is a common target for extra articular affection in RA patients. Both stem cell and bevacizumab are promising lines of treatment, but further studies on a larger number of rats and even on human after full research on rats are highly needed.

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