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# Assessment of the effects of mesenchymal stem cells-derived microvesicles on rheumatoid arthritic male rats

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**Abstract**

**Background:** Rheumatoid arthritis (RA) is a chronic, progressive, autoimmune disease caused by a malfunction of the immune system. The aim of this study was to examine the anti-arthritic effects of mesenchymal stem cell (MSC) derived microvesicles (MVs). **Method**: Thirty male rats were divided into 3 equal groups (control, RA, and RA treated with microvesicles groups). Complete Freund's adjuvant (CFA) was used to establish RA in the animals by subcutaneous injection of 100 µL CFA/rat into plantar region of right hind leg in two consecutive days. RA rats were intravenously injected with MVs (100 µg protein/rat/week) into lateral tail vein of CFA-induced arthritic rats for 3 weeks. **Results:** CFA injection was deleterious to the ankle joints. CFA-induced arthritic rats showed high levels of serum pro-inflammatory

mediators, such as rheumatoid factor (RF), tumour necrosis-alpha (TNF-α), and interleukin-1β (IL-1β). MVs treatment significantly lowered these pro-inflammatory mediators and increased serum level of anti-inflammatory cytokine, IL-10. **Conclusion:** MSC MVs exerted anti-arthritic and anti-inflammatory effects in CFA-induced arthritic rats.

## 1. Introduction:

Rheumatoid arthritis (RA) is an inflammatory synovitis-associated chronic systemic autoimmune disease that primarily damages the synovial tissue of the joints. It is characterized by an increase in the inflammatory proteins interleukin (IL) and tumor necrosis factor (TNF), as well as the activation of T cells, which can cause severe, persistent joint inflammation and potentially the erosion and destruction of cartilage, bone, and joints **(1).**

The early stages of RA are characterized by early reactivity with a small number of self-antigens, limited systemic inflammation, and tissue injury. After a certain threshold is crossed, clinically apparent IA (inflammatory arthritis)/RA develops. Anti-citrullinated protein antibodies (ACPA) data showing a rise in both quantity and type over time have been used to support this idea **(2).**

Patients with RA typically suffer from symmetrical joint swelling, morning stiffness and pain caused by an inflammatory synovitis. RA usually involves the small joints of the fingers and toes, preferentially inflaming the proximal interphalangeal and metacarpophalangeal joints, but also wrists, ankles, and knees **(3)**.

Rheumatologists used the morning stiffness in addition to test data and swollen or painful joints to classify the illness. Morning joint pain and indicators of disease activity only weakly correlate with the "severity" of morning stiffness, which is substantially connected with morning joint pain (**4**).

The disease's progression and joint degeneration can be slowed down or prevented with treatment for RA. Leech therapy, physical activity, massage therapy, acupuncture, and surgical procedures like arthroscopy are

examples of non-pharmacological treatment options **(5).**

Despite the fact that there is currently no cure for rheumatoid arthritis, there are medications that can help control discomfort while also delaying the disease's progression and further joint damage. By preventing the formation of prostaglandins, nonsteroidal anti-inflammatory medications (NSAIDs) may lessen inflammation. but they do not stop joint deterioration. Glucocorticoids, like NSAIDs, reduce inflammation without effecting disease progression but through the reduction of pro-inflammatory cytokines. Disease-modifying antirheumatic drugs (DMARDs) prevent further disease progression and inflammation while contributing to joint repair but they have aggressive side effects. Tumor necrosis factor (TNF)-α inhibitors are used in the form of injections to manage the chronic inflammation that results from RA **(6)**

Lipid-bound vesicles called extracellular vesicles (EVs) are secreted into the extracellular space by cells. Microvesicles (MVs), exosomes, and apoptotic bodies are the three primary subtypes of EVs, and they are distinguished by their biogenesis, release mechanisms, size, composition, and function **(7)**.

The study of EVs has contributed to a greater knowledge of cancer metastasis and cell-cell communication, and it has also demonstrated the potential of EVs in the clinical context as carriers of biomarkers for diagnostic purposes **(8).**

The aim of this study is to assess the anti-arthritic effects and to suggest the mechanisms of action of mesenchymal stem cell (MSC) derived microvesicles (MVs) on arthritic induced male rats.

## 2. Materials and Methods:

### I-Experimental Animals

Thirty Wistar male rats weighing about 100-120 g were used as experimental animals in the present investigation. The chosen animals were housed (four per cage) in polystyrene cages with good, aerated covers in the animal house of Zoology Department, Faculty of Science, Beni-Suef University, Egypt at normal temperature (25±5°) as well as with a 12-hour light/dark cycle. Moreover, they were given access of water and supplied daily with standard diet of known composition. All experimental protocols described in the present study were approved by the

Ethics Review Committee for Animal Experimentation of Beni-Suef University, Egypt (Ethical approval number: (022-231)).

**II-Drug Preparation**

Complete Freund's adjuvant (CFA) was purchased from Sigma Chemicals Co., St. Louis, MO, USA, stored at 2-4 °C and protected from sunlight. All other chemicals used in this investigation were of analytical grade and were obtained from standard commercial supplies.

**III - Induction of Rheumatoid Arthritis**

Adjuvant arthritis was induced by two subcutaneous injections of 0.1 ml complete Freund's adjuvant (CFA), suspension of heat-killed *Mycobacterium tuberculosis* in mineral oil, into a footpad of the right hind leg of male rats in two conscious days (**9**).

**IV-MSC microvesicles isolation:**

MSCs were seeded at 6 $x10^4$ cells/$cm^2$ in proliferative medium for 24 h and then with production medium for 48 h. Condition media harvested from MSCs is first centrifuged at 300 ×g for 10 minutes. Next, the supernatant is ultracentrifuged at 16,500 ×g for 20 minutes following by a washing step. Finally, the MVs pellet is washed and resuspended in PBS and kept at -80 °C **(9).**

**V- Animals Grouping**

The considered rats were divided equally into three groups each contains 10 rats as following:

**1- Group 1 (Control group):**

It included 10 male rats and was regarded as normal animals which were given ,1ml saline IV daily for 3weeks and kept under the same laboratory conditions and was regarded as normal control group for other ones.

**2- Group 2 (RA group):**

Rats were given and were kept under the same laboratory conditions intravenously (IV) given 0. 1 mL saline/rat/week for 3 weeks.

**3- Group 3 (RA treated with MVs):**

RA rats were injected IV with MSC MVs (100 µg in 0.1 mL saline /rat/week) into lateral tail vein for 3 weeks **(8).**

At the end of the experiment, rats of each group were sacrificed under diethyl ether anesthesia and blood was collected from jugular vein. Serum was separated by centrifugation of blood at 3000 rpm for 15 minutes and the clear non-hemolyzed supernatant sera were quickly removed, divided into three

portions for each individual animal, and kept at -20°C till used.

**VI- Experimental Studies**

**VI- 1- Determination of serum RF concentration:**

The level of RF in the serum of control and experimental groups were determined using specific enzyme-linked immunosorbent assay (ELISA) kits purchased from R and A systems, USA according to manufacturer's instructions.

**VI- 2- Determination of serum Anti-CCP concentration:**

The level of serum Anti-CCP in the serum of control and experimental groups were determined using specific enzyme-linked immunosorbent assay (ELISA) kits purchased from R and A systems, USA according to manufacturer's instructions

**VI- 3- Determination of serum TNF-α concentration:**

The level of serum TNF-α was determined using specific ELISA kits purchased from R and A systems, USA according to manufacturer's instructions.

**VI- 4- Determination of serum IL-1β concentration:**

Serum IL-1β was determined by ELISA kits obtained from Thermo Scientific (USA) according to manufacturer's instructions.

**VI-6- Determination of serum IL-10 concentration:**

The level of serum IL-10 was determined using specific ELISA kits purchased from R and A systems, USA.

**VII- Statistical Analysis**

Data were coded and entered using the statistical package SPSS version 23. Data was summarized using mean and standard deviation for quantitative variables. Analysis of variance (ANOVA) with multiple comparisons post hoc test was applied for comparing more than 2 groups. P-values less than 0.05 were considered as statistically significant.

## 3. Results:

**I. Effect on serum RF: Table (1)** and **figure (1)**

Serum RF was statistically significant (p-value <0.001) higher by + 348.16 % in group 2 in comparison to group 1. However, serum RF showed a statistical significant (p-value <0.001) decrease (- 40.61%) in group 3 when compared to group 2 but still higher than group 1.

**Table (1): Comparison of RF level in the studied groups.**

| | Group 1 | Group 2 | % Change | Group 3 | % Change | p-value |
|---|---|---|---|---|---|---|
| **RF (IU/mL)** | 27.2 ±3.7 | 121.9[a] ±22.7 | + 348.16 % | 72.4[a,b] ± 12.2 | - 40.61% | <0.001 |

*Values are presented as mean ± SD*
*a: significance from group 1 (control) at p-value <0.05*
*b: significance from group 2 (diseased) at p-value <0.05*

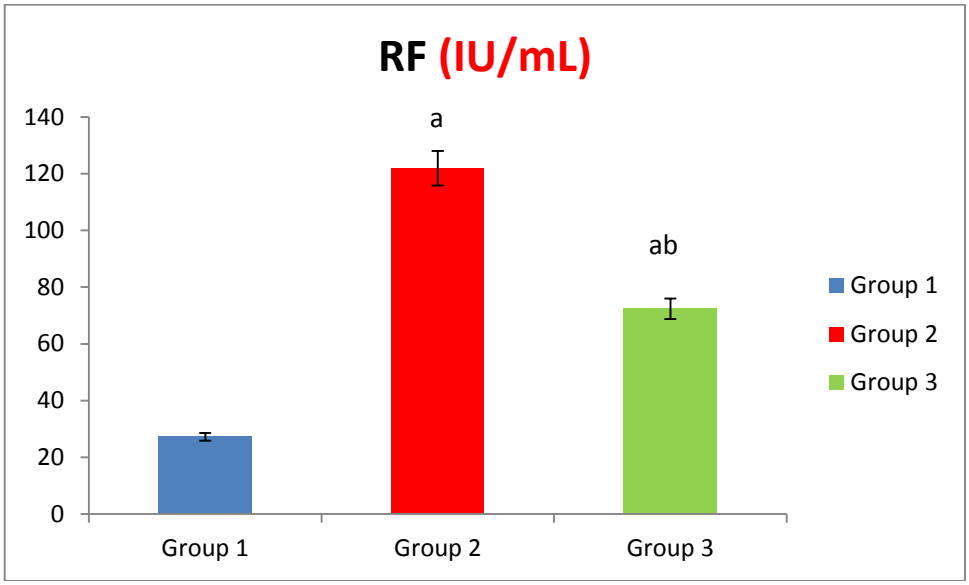


**Figure (1): Comparison of RF level in different study groups.**

*Values are presented as mean ± SD*
*a: significance from group 1 (control) at p-value <0.05*
*b: significance from group 2 (diseased) at p-value <0.05*

**II. Effect on serum anti-CCP: Table (2)** and **figure (2)**

Serum anti-CCP was statistically significant (p-value <0.001) higher by + 345.88 % in group 2 in comparison to group 1. However, serum anti-CCP showed a statistical significant (p-value <0.001) decrease (- 36.93 %) in group 3 in comparison to group 2.

**Table (2): Comparison of Anti-CCP level in the studied groups:**

| | Group 1 | Group 2 | | Group 3 | | p-value |
|---|---|---|---|---|---|---|
| **Anti-CCP level (IU/mL)** | 8.5±1.6 | 37.9 [a] ±2.9 | +345.88% | 23.9 [a,b] ±3.7 | - 36,93 % | <0.001 |

*Values are presented as mean ± SD*
*a: significance from group 1 (control) at p-value <0.05*
*b: significance from group 2 (diseased) at p-value <0.05*

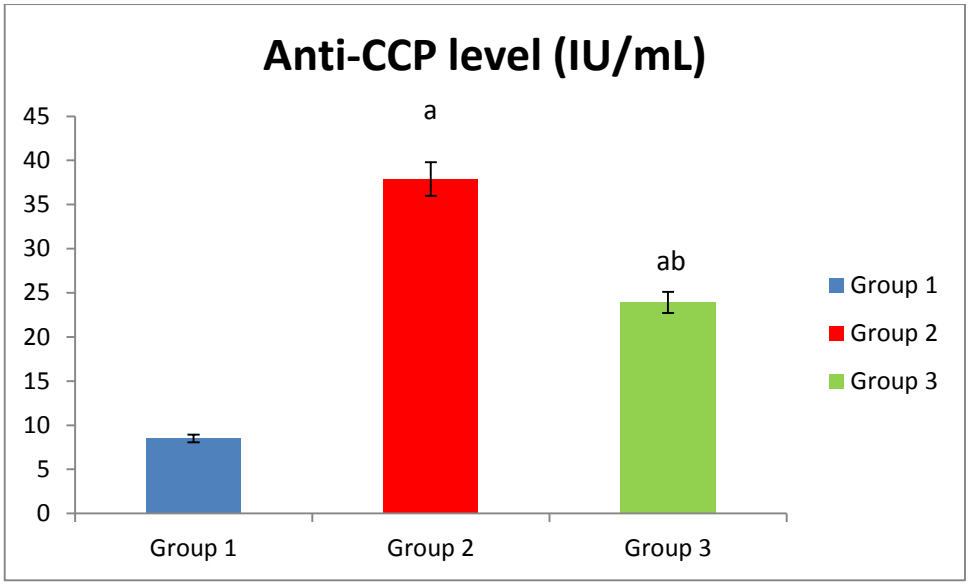


**Figure (2): serum anti-CCP in the studied groups.**

*Values are presented as mean ± SD*
*a: significance from group 1 (control) at p-value <0.05*
*b: significance from group 2 (diseased) at p-value <0.05*

**III. Effect on serum Pro-inflammatory markers: Table (3)** and **figures (3, 4)**

Serum pro-inflammatory markers (TNF-α and IL-1β) showed significant increase p-value <0.001 in group 2 in comparison to group 1 by 420.37% and 488.98% respectively.

However, there was a statistically significant (p-value <0.001) decrease (-50.63%) in serum TNF-α level in group 3 in comparison to group 2 and also in IL-1β level in group 3 by -47.72%.

**Table (3): Comparison of TNF- α and IL-1β levels in different study groups.**

| | **Group 1** | **Group 2** | | **Group 3** | | **p-value** |
|---|---|---|---|---|---|---|
| **TNF- α (pg/mL)** | 21.1±3.8 | 109.8 [a] ±17.6 | 420.37% | 54.2 [a,b] ±7.5, | -50.63% | <0.001 |
| **IL-1β (pg/mL)** | 22.7±4.1 | 133.7 [a] ±11.4 | 488.98% | 69.9 [a,b] ±13.4 | -47.72 | <0.001 |

*Values are presented as mean ± SD*
*a: significance from group 1 (control) at p-value <0.05*
*b: significance from group 2 (diseased) at p-value <0.05*

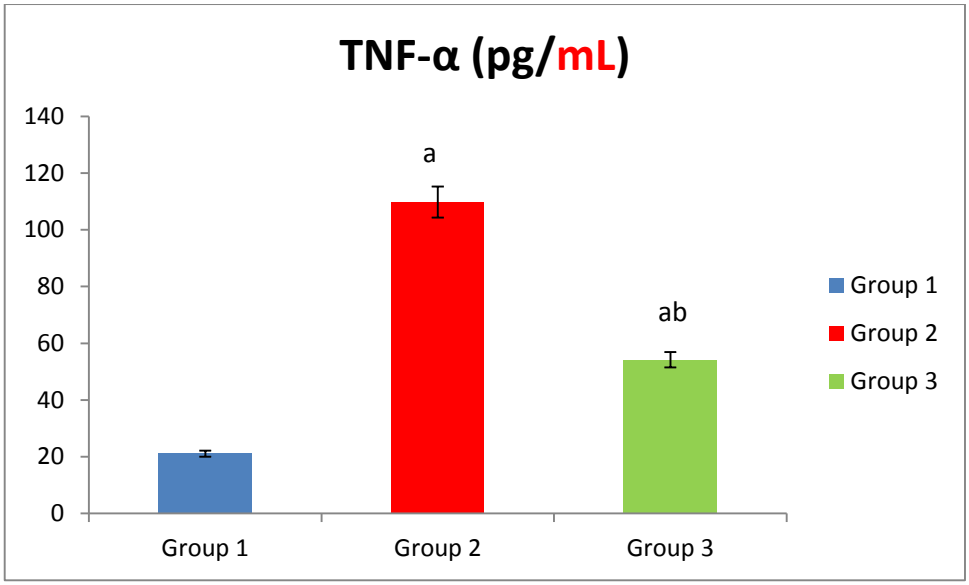


**Figure (3): serum TNF-α in the studied groups.**

*Values are presented as mean ± SD*
*a: significance from group 1 (control) at p-value <0.05*
*b: significance from group 2 (diseased) at p-value <0.05*

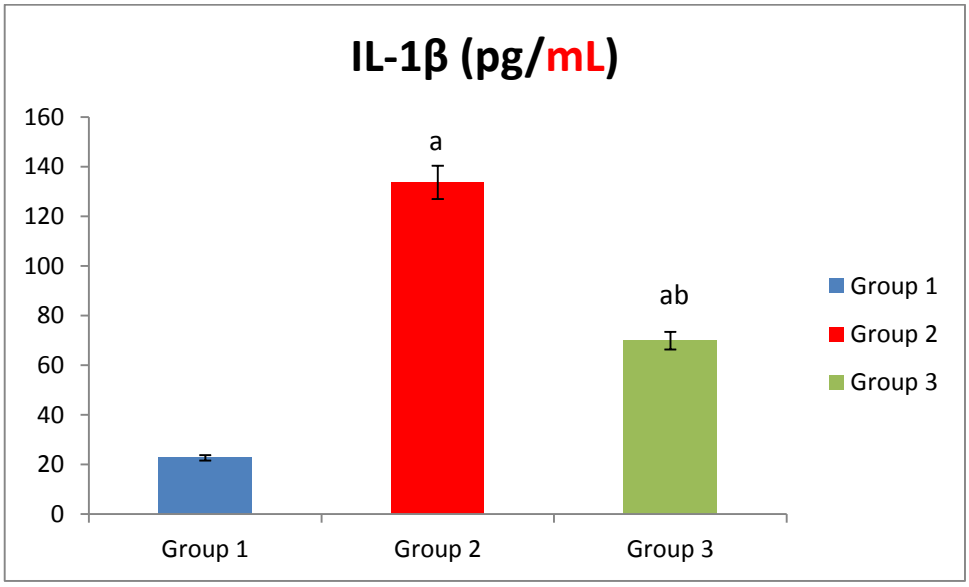


**Figure (4): serum IL-1β in studied groups.**

*Values are presented as mean ± SD*
*a: significance from group 1 (control) at p-value <0.05*
*b: significance from group 2 (diseased) at p-value <0.05*

**IV. Effect of serum anti-inflammatory marker (IL-10): Table (4)** and **figure (5)**

Serum IL-10 showed a significant decrease (p-value <0.001) in group 2 in comparison to group 1 by -76.78%.

However, there was a statistically significant (p-value <0.001) increase (102%) in serum IL-10 level in group 3 in comparison to group 2.

**Table (4): Comparison of IL-10 level in the studied groups:**

| | Group 1 | Group 2 | | Group 3 | | p-value |
|---|---|---|---|---|---|---|
| **IL-10 level (pg/ml)** | 292.4 ±17.9 | 67.9 [a] ±11.6 | -76.78% | 137.8 [a,b] ±13.3 | 102% | <0.001 |

*Values are presented as mean ± SD*
*a: significance from group 1 (control) at p-value <0.05*
*b: significance from group 2 (diseased) at p-value <0.05*

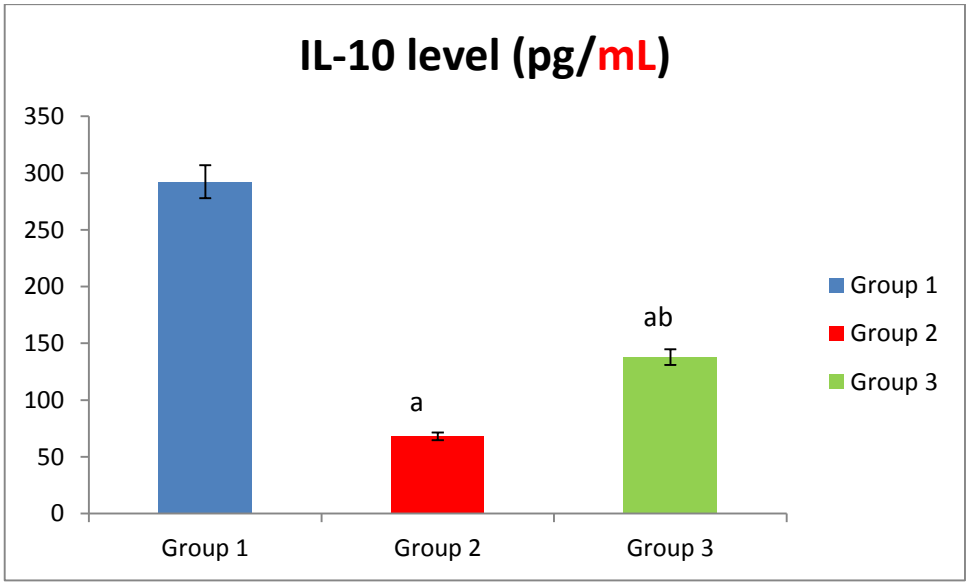


**Figure (5) serum level IL10 in studied groups.**

*Values are presented as mean ± SD*
*a: significance from group 1 (control) at p-value <0.05*
*b: significance from group 2 (diseased) at p-value <0.05*

## 4. Discussion:

(RA) is a typical chronic autoimmune illness that has a high mortality rate, significant morbidity, and annual expenses in the billions of dollars **(10)**

Early stages of RA are characterised by early reactivity with a small number of self-antigens, as well as a small amount of systemic inflammation. These early stages are then followed by the evolution over time of expanding innate and adaptive responses, as well as tissue injury, as well as the expansion of other autoantibody systems and a larger amount of systemic inflammation (e.g., cytokines) **(2)**

MSCs are capable of differentiating into chondrocytes, adipocytes, and osteoblasts. The release of a wide range of trophic factors, such as growth factors, cytokines, and chemokines, allows MSCs to perform a number of other tasks besides their capacity for differentiation, such as anti-inflammatory, proliferative, anti-apoptotic, anti-fibrotic, and angiogenic capabilities **(11).**

(CFA) in Wistar rat model was used in this study because CFA has been known to cause a series of inflammatory reactions like in humans. The injection of CFA at the rat's footpad results in the cutaneous inflammation presented as reddish and swollen ankle **(12**).

The present study was designed to evaluate the efficacy of the use of MSC MVs for 3 weeks on serum levels of RF, anti-CCP, pro-inflammatory biomarkers (IL-1 β and TNF-α), and serum anti-inflammatory cytokine (IL-10).

The present data revealed that use of micovesicles caused a significant amelioration of serum pro-inflammatory cytokines and joint damage of adjuvant-induced rheumatoid arthritis in male rats. Thus, this study provides the evidence that MSCs-derived micovesicles exert an immunomodulatory effect in a rheumatoid arthritis model.

The current study revealed a significant increase in serum RF and serum Anti-CCP levels in group 2 (RA group) in comparison to group 1 (control group). This is in line with Sameh et al. **(13)** who proved that anti Anti-CCP antibodies have been proven to accurately predict both the onset of RA and the extent of concomitant joint damage.

In addition, our result is in agreement with a previous study which showed that acute exacerbation of RA, RA patients frequently show high titers

of autoantibodies including RF and anti-CCP antibody **(14)**.

However, serum RF and serum Anti-CCP showed a statistical significance decrease in group 3 (RA treated with MVs) in comparison to group 2 (RA group) but still higher than group 1(control group). This can be explained by that MSCs from healthy donors have been found to suppress B cell proliferation so decrease anti-ACPA and RF production **(13)**

Pro-inflammatory T cells, cytokines, and immune complexes drive macrophages and fibroblast-like synoviocytes (FLS) to release pro-inflammatory cytokines such TNF-, IL-1, IL-6, IL-15, and IL-23. Inflammation is a complex pathophysiological process. **(15).**

Cyclooxygenase (COX) is strongly induced by IL-1 and plays an important role in the pathophysiology of RA; therefore, down-regulation of pro-inflammatory cytokines and NF-B may be an appropriate therapeutic strategy for RA. Synovial fibroblasts respond vigorously to pro-inflammatory cytokines and form pannus tissues, which destroy the cartilage and bone of the joints (**16**).

The current study revealed increased serum levels of pro-inflammatory markers (IL-1β and TNF-$\alpha$) in the rheumatoid arthritis group in comparison with the normal control group. These results run parallel to previous study that showed that progression of RA is accelerated by pro-inflammatory cytokines and chemokines including TNF-α and IL-1β **(17).**

In comparison to the arthritic control, the serum pro-inflammatory markers (IL-1β and TNF-$\alpha$) showed a a significant decrease in group 3 (RA treated with MVs). This is in line with the study of Kyurkchiev et al. (**18)**, who found that MVs have impact on immune system, cytokines/growth factors as well as other cytokines such as TNF-α, IL-1β, and IL-17. However, in contrast to our result, Conforti et al. **(19)** demonstrated that there is very low effect of MVs in reduction of pro-inflammatory markers (TNF α and IL-1β).

In addition, RA model rats showed obviously enhanced limb swelling, and promoted inflammation in synovial tissues, and TNF-α, IL-6 and IL-8 expression was notably enhanced both in vivo and in vitro; however, BM-MSCs-derived EV transfection reversed these symptoms in the RA model rats **(20).**

In RA synovial membranes, a compensatory anti-inflammatory

response is also seen. An upstream regulator and anti-inflammatory marker called IL-10 is hypothesized to adversely influence RA progression. Numerous research using animal models of arthritis have demonstrated the positive effects of IL-10 on reducing arthritis severity **(21).**

The current study revealed significant decreased serum levels of anti-inflammatory marker, IL-10, which showed significant decrease in group 2 in comparison to group 1 (control group).

However, serum IL10 showed a significant increase in group 3 (RA treated with MVs) in comparison to group 2 (RA group) but still lower than group 1 (control group).

Our results are parallel to Ahmed et al. **(22),** who proved that there is decrease in IL10 in RA rats in comparison to other treated groups.

MSCs exert potent immunomodulatory and anti-inflammatory actions by cell-to-cell interactions between MSCs and lymphocytes or by soluble factor production **(23).**

This is also consistent with earlier research, which found that the immunosuppressive, antifibrotic, and anti-inflammatory properties of MSC-derived EVs could lower inflammation in RA **(11).**

However, other studies have shown that MVs produced from MSCs are less effective than MSCs alone in stimulating the synthesis of TGF and IL-10 in B and T cells **(24).**

In conclusion, MSC MVs have potent anti-arthritic effects in CFA-induced arthritic rats, which may be mediated *via* their anti-inflammatory effects. However, further studies are required to elucidate the mechanisms of actions of MSC MVs to produce the anti-arthritic effects. Further clinical studies are also required to assess the efficacy and safety of MSCs MVs on RA in human beings

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