

Research Article

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Conditioned Medium from Estradiol-Primed Macrophages Mitigates Adjuvant-Induced Arthritis in Rats

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ABSTRACT

**Purpose:** Macrophages with an anti-inflammatory phenotype are critical for resolving inflammation and preventing chronic tissue injury. Estradiol is known to promote this favorable macrophage profile. This study evaluated the therapeutic potential of the secretome, delivered as conditioned medium, from estradiol-treated macrophages in experimental rheumatoid arthritis in Wistar rats.

**Methods:** Rheumatoid arthritis was induced in Wistar rats using complete Freund's adjuvant. Animals were assigned to five groups: healthy controls, arthritic rats receiving vehicle, arthritic rats treated with prednisolone, arthritic rats treated with conditioned medium from untreated macrophages, and arthritic rats treated with conditioned medium from estradiol-exposed macrophages. The lyophilized media were administered intraperitoneally on days 4, 12, and 20 post-induction; the study ended on day 24.

**Results:** Conditioned medium from estradiol-treated macrophages exhibited significantly higher levels of anti-inflammatory mediators such as Interleukin-10, Transforming Growth Factor-beta, and Indoleamine 2,3-dioxygenase, along with increased messenger RNA expression of regulatory genes including Early Growth Response 2 and Mannose Receptor. In vivo, this treatment notably reduced arthritis severity and improved weight gain compared to medium from untreated macrophages. These effects correlated with a marked decrease in antigen-specific proliferation and serum levels of inflammatory markers such as C-reactive protein, Myeloperoxidase, Nitric oxide, Interleukin-1, and Tumor Necrosis Factor-alpha. Additionally, bone-destructive factors like Receptor Activator of Nuclear Factor Kappa-B Ligand and Matrix Metalloproteinase-9 were significantly downregulated in treated rats.

**Conclusion:** The conditioned medium derived from estradiol-treated macrophages, enriched with anti-inflammatory and regulatory components, presents a promising cell-free therapeutic strategy for immunotherapy in rheumatoid arthritis.

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## 1. Introduction

One of the autoimmune diseases is rheumatoid arthritis (RA) characterized by joint inflammation, affecting approximately 1% of the global population. It is 2 to 3 times more common in women than men and can manifest at any age.<sup>1,2</sup> The disease typically begins with progressive inflammation in multiple joints of the hands and feet, leading to pain and restricted movement.<sup>3-6</sup> If the disease is not well controlled, it may cause extra-articular complications such as small vessel vasculitis, keratitis, pulmonary granulomas, as well as pericarditis and pleuritis.<sup>7</sup> Some patients do not respond well to current treatments due to the disease's complex immunopathology, and these treatments can have serious side effects. Consequently, research is ongoing to find new therapeutic approaches for these patients.<sup>8</sup>

While the immune system is essential for survival and health, its potential for harm highlights the complexity and importance of maintaining a balanced immune response. This "double-edged sword" property illustrates both the advantages and risks associated with immune activity.<sup>9</sup> CD4+ lymphocytes and macrophages (MΦ) are crucial in immunopathological conditions like rheumatoid arthritis; they mediate and regulate immune responses, making them vital for understanding these processes.<sup>10</sup> Macrophages are versatile and critical for both immediate defense against pathogens and for shaping the longer-lasting adaptive immune response. The immune system's characteristic double-edged sword effect is primarily due to the specialized function of macrophages, which can be activated to initiate either a restorative, anti-inflammatory (M2) response or an aggressive, inflammatory (M1) reaction.<sup>9,11-13</sup> Anti-inflammatory macrophages (M2) are essential for a balanced immune system, supporting tissue repair and regeneration. They can transition into M1 macrophages when facing threats like pathogens or abnormal cells. While this transition is important, an excessive M2 macrophage response can cause complications such as persistent infections, fibrosis, allergic reactions, and tumor growth.<sup>9,14</sup> In contrast, M1 macrophages are mainly linked to autoimmune responses, atherosclerosis, and chronic inflammation, underscoring their varied roles in immune-related disorders.<sup>14,15</sup>

Many macrophage functions are mediated by soluble factors. Macrophage-conditioned medium (MΦCM) refers to the culture medium enriched with factors secreted by macrophages during in vitro growth after stimulation. When cultured, macrophages release bioactive factors such as chemokines and cytokines, as well as extracellular matrix components and growth factors into the medium.<sup>16,17</sup> MΦCM provides a powerful model for understanding the myriad ways in which these cells communicate and coordinate immune responses. MΦCM research can help uncover potential therapeutic targets for enhancing immunity or suppressing unwanted inflammation in various diseases.<sup>18,19</sup> MΦCM has been utilized in the treatment and experimental modeling of various diseases, particularly in cancer research and immune response studies.<sup>19-21</sup>

17β-Estradiol (E2) is a potent estrogen that influences macrophage function and polarization through estrogen receptors (ERs), primarily ERα and ERβ. It impacts immune responses, especially in asthma and autoimmune diseases. Female-derived macrophages demonstrate higher M2 polarization due to estradiol, enhancing anti-inflammatory responses compared to male-derived macrophages.<sup>22</sup> Estradiol has been shown to affect the polarization of macrophages, promoting an anti-inflammatory M2 phenotype while inhibiting the pro-inflammatory M1 phenotype.<sup>23,24</sup> Estradiol treatment has also been reported to increase anti-inflammatory markers like IL-10 and reduce pro-inflammatory cytokines such as IL-6 and TNF-α in macrophages. This modulation may help improve immunopathological conditions like RA.<sup>23,25</sup> Despite the promising potential of conditioned medium derived from E2-primed macrophages in managing RA, much research is still needed to discover its therapeutic

benefits. This research seeks to address this gap by focusing on the effects of conditioned medium obtained from E2-pulsed peritoneal macrophages on controlling experimental rheumatoid arthritis in Wistar rats.

## 2. Material and Methods

### 2.1. Macrophage Isolation and Conditioned Medium Preparation

Peritoneal macrophages were isolated by injecting 20 ml of cold PBS (4°C) into peritoneal cavity of Wistar rats as previously described.<sup>13</sup> To begin, peritoneal fluid collection was performed, followed by centrifugation for 10 minutes at 600 g (4°C). The cellular pellets were then twice rinsed with PBS and reconstituted in DMEM with 10% heat-inactivated Fetal Bovine Serum (FBS). For optimal macrophage adherence, a cellular suspension containing  $2 \times 10^6$  viable cells/mL was pre-incubated for 40 minutes in 48-well microplates (37°C), maintaining a humidified environment with 5% CO<sub>2</sub>. Unadhered cells were eliminated by triple washing with cold PBS (4°C). Cell viability, assessed through trypan blue exclusion, consistently remained above 96%.

Afterward, macrophages were treated with either 0 or 100 nM 17 $\beta$ -estradiol for 24 hours. After the medium was aspirated, the cells underwent three washes with PBS. Subsequently, the macrophages were then maintained in serum-free DMEM for another 24 hours. Conditioned medium was harvested, subjected to centrifugation at 300 g for 10 minutes, and subsequently passed through a 0.2- $\mu$ m filter to remove cellular debris.

Using ELISA kits from Bender MedSystems, Austria, and according to the manufacturer's instructions, the concentrations of TGF- $\beta$  and IL-10 in the conditioned medium were determined. After isolating the conditioned medium, macrophages yielded total RNA, which was then extracted using the Trizol method and converted into complementary DNA. The mRNA expression of mannose receptor (MR) and Early Growth Response Gene-2 (EGR2) was analyzed, with GAPDH serving as an internal control. For quantification of target gene mRNA levels, SYBR green mix was used, with results reported as fold change ( $2^{-\Delta\Delta C_t}$ ) from at least three distinct experiments. The amplification primer sequences are provided in Table 1.

**Table 1.** The sequence of primers.

| Gene         | Forward Sequence           | Reverse Primer             |
|--------------|----------------------------|----------------------------|
| <b>MR</b>    | 5'-ATGGCCTTCCTGGTGCTCT-3'  | 5'-TCAGGCACAGCTTCCACATC-3' |
| <b>EGR2</b>  | 5'-CAGCCGAGCCATGAACATC-3'  | 5'-GCTGGTGTTGGTGTGATG-3'   |
| <b>RANKL</b> | 5'-ATGCGGTGAGCTACAGGATG-3' | 5'-TTCAGGAGGATTGAGCTGGA-3' |
| <b>MMP9</b>  | 5'-AGCACTGTGTGCCTTTACCC-3' | 5'-CCAGCCAGTCTGAGTCTTCA-3' |
| <b>GAPDH</b> | 5'-GACAGTCAGCCGCATCTTCT-3' | 5'-TGTAGTTGAGGTCGGTGTGA-3' |

Additionally, indoleamine-2,3-dioxygenase (IDO) activity was assessed by a kynurenine detection assay, as described previously.<sup>9</sup> Briefly, 100  $\mu$ L of sample received 50  $\mu$ L of trichloroacetic acid (30%), after which the mixture was vortexed and centrifuged for 5 minutes at  $10,000 \times g$ . Subsequently, in a 96-well microtiter plate, 75  $\mu$ L of the resulting supernatant was intermixed with 75  $\mu$ L of Ehrlich's reagent (containing 100 mg p-dimethylaminobenzaldehyde in 5 mL glacial acetic acid). Measurement of optical density was performed at 492 nm utilizing a microplate reader. To ascertain unknown concentrations, a standard curve encompassing kynurenine concentrations from 0 to 100  $\mu$ M was employed.

For the animal study phase, the isolated conditioned media were lyophilized via a Christ Alpha1-2 LD Plus freeze dryer (Germany) and were subsequently enriched 50-fold from their initial concentration.

## 2.2. Animals

Male Wistar rats, weighing 160-180 g, were procured from the Faculty of Veterinary Medicine at our university. They were maintained under standardized conditions (55%  $\pm$  5% humidity, 20°-25°C and 12 h light/dark cycle) with unrestricted access to food and water, in accordance with the Helsinki Convention and Iranian Ministry of Health regulations.

## 2.3. RA Induction, Monitoring, and Treatment

Induction of RA involved the intradermal injection of 0.1 mL of complete Freund's adjuvant (CFA), formulated with 10 mg/mL of inactivated Mycobacterium, into the hind paw. An electronic water plethysmograph facilitated the measurement of non-injected hind paw volume, specifically up to the anatomical hairline of the lateral malleolus. A scoring system was employed to assess disease severity: 4 for full leg swelling and loss of flexibility; 3 for ankle swelling; 2 for erythema and paw swelling; 1 for toe erythema; and 0 for a normal paw.<sup>26-28</sup>

Three independent observers conducted assessments each morning during the study, subsequently reporting the average scores. Only the non-injected paws were evaluated for arthritis, yielding a maximum score of 12. Furthermore, the weight changes of each rat were documented every other day following immunization. Therapy commenced on day 4, coinciding with the onset of RA signs in all rats, and continued until day 24 post-induction, when the animals were sacrificed. At this stage, Animals were then randomly divided into five groups of 10 rats each: RA rats received with Vehicle, RA rats treated with lyophilized macrophage conditioned medium without E2 treatment (M $\phi$ CM), RA rats treated with lyophilized conditioned medium isolated from E2-pulsed macrophages (M $\phi$ CM-E2), RA rats receiving prednisolone, and healthy control rats.

The lyophilized conditioned media employed in the animal study possessed a final protein concentration of 10 mg/mL. The administration of M $\phi$ CM and M $\phi$ CM-E2 was performed intraperitoneally on days 4, 12, and 20, using a volume of 1 mL for each treatment. Additionally, prednisolone (2 mg/kg) was administered daily via intraperitoneal injection throughout the treatment period. In contrast, the healthy control and vehicle-treated RA rats were given an equivalent volume of phosphate-buffered saline (PBS) to maintain comparability.

## 2.4. Serum Biochemical Evaluation

Cardiac blood samples, collected before sacrifice, underwent centrifugation for 10 minutes at 4,000 rpm (4 °C). Serum was subsequently isolated and stored at -70 °C until further analysis. Utilizing ELISA kits (BD, UK), and adhering to the manufacturer's instructions, serum TNF- $\alpha$  and IL-1 $\beta$  levels were determined. The level of Nitric oxide (NO) in the serum was measured immediately after serum separation, utilizing the Griess colorimetric method, as described previously.<sup>29</sup> addition, commercial kits from (BD, UK), were employed to ascertain C-reactive protein (CRP) concentrations, as per the manufacturer's guidelines. This comprehensive approach enabled the evaluation of the inflammatory and oxidative stress status of the serum samples through the quantification of MPO activity, nitric oxide levels, and CRP concentrations.

As previously detailed, the myeloperoxidase (MPO) activity in isolated serum samples was evaluated.<sup>30</sup> For the assay, 10  $\mu$ L of serum was mixed with 80  $\mu$ L of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and 0.75 mM of TMB solution (3,3',5,5'-tetramethylbenzidine). The TMB solution was derived from a stock containing 150 mM phosphate buffer at pH 5.4, 2.9 mM TMB and 14.5% DMSO. Following this, the mixture underwent a 5-minute incubation at 37 °C to facilitate the reaction. Reaction termination was achieved by adding 50  $\mu$ L of 2 M sulfuric acid to each

well of the assay plate. After an additional 5 minutes, the absorbance was measured at 450 nm using an ELISA reader. MPO activity results were quantified and expressed in units per liter (U/L).

### 2.5. Splenocyte Proliferation Assay

In the splenocyte proliferation assay, spleens from each rat were dissected and placed in 5 mL of DMEM containing 10% FBS. Cellular material was isolated by passage through a 100  $\mu$ m nylon mesh and subjected to centrifugation at 200 g for 10 minutes. To deplete red blood cells, ACK-lysing buffer was introduced to the pellet. In a 96-well plate, cells were prepared as a 100  $\mu$ L suspension ( $2 \times 10^5$  cells/well) and then challenged with 100  $\mu$ g/mL whole mycobacterial antigen (*Mtb*). Each experiment was performed in triplicate. After 72 hours, 25  $\mu$ L/well of MTT solution (5 mg/mL) was supplemented to the culture 4 hours before its conclusion. Lymphocytes were centrifuged for 10 minutes at 1000 g, and DMSO (150  $\mu$ L) was added for the dissolution of formazan crystals. Absorbance at 570 nm was recorded, and the stimulation index was calculated by applying the formula:  $[\text{OD (with } Mtb) - \text{OD (blank)}] / [\text{OD (without } Mtb) - \text{OD (blank)}]$ .<sup>10</sup>

### 2.6. Determination of the mRNA Expression of MMP-9 and RANKL

Quantitative real-time RT-PCR (qRT-PCR) necessitated the extraction of total RNA from ankle joint tissue using liquid nitrogen pulverization followed by the Trizol method, and its subsequent conversion into complementary DNA. The mRNA expression of MMP-9 (Matrix Metalloproteinase-9) and RANKL (Receptor Activator of Nuclear Factor Kappa-B Ligand) was analyzed, with GAPDH serving as an internal control. For the quantification of target gene mRNA levels, SYBR green mix was employed, with results expressed as fold change ( $2^{-\Delta\Delta C_t}$ ) derived from a minimum of three independent experiments. Table 1 provides the primer sequences used for amplification.

### 2.7. Statistical Analysis

SPSS version 21 was utilized for statistical analysis, with data presented as Mean  $\pm$  SD. Analysis of nonparametric data (arthritis index) was performed using the Kruskal-Wallis test. This was subsequently followed by Mann-Whitney U evaluation, incorporating Bonferroni adjustment. Remaining parametric data were evaluated using one-way ANOVA and Tukey's post-hoc test, with a *P*-value of  $< 0.05$  considered statistically significant.

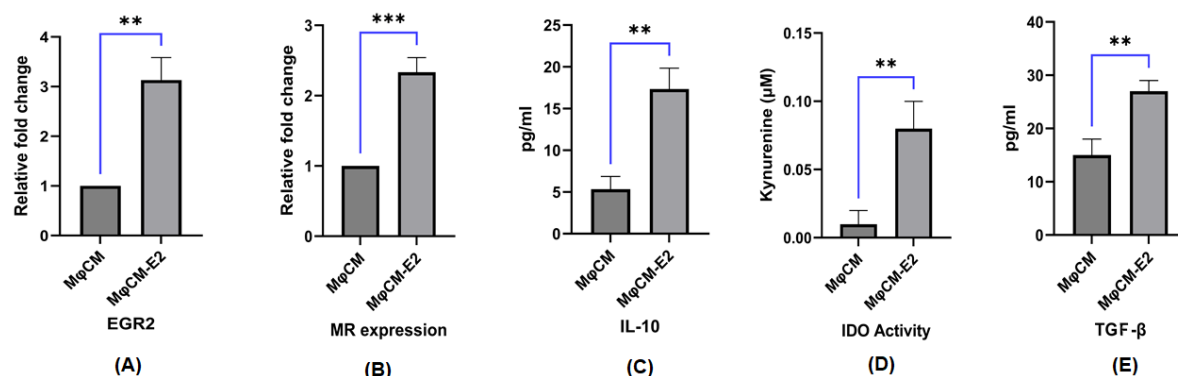
## 3. Results

### 3.1. Initial Assessment of E2-Treated Macrophages: Gene Expression and Secretion Profile

RT-PCR analysis confirmed that E2 treatment significantly augmented the mRNA expression of key genes in macrophages (Figure 1A and 1B). Specifically, EGR2 mRNA levels demonstrated a substantial 3.1-fold increase in M $\phi$ CM-E2 compared to untreated M $\phi$ CM (Relative fold change:  $3.14 \pm 0.35$  vs.  $1.00 \pm 0.00$ ;  $P < 0.01$ ). Concurrently, MR mRNA expression also exhibited a significant increase of approximately 2.3-fold following E2 stimulation (Relative fold change:  $2.30 \pm 0.28$  vs.  $1.00 \pm 0.00$ ;  $P < 0.001$ ). These findings indicate a clear transcriptional modulation by E2 towards a specific macrophage phenotype.

Further investigation of the conditioned medium from these macrophages revealed a marked shift towards an anti-inflammatory and immunomodulatory profile. We observed a significant increase in the levels of IL-10 ( $17.52 \pm 2.45$  pg/ml) in M $\phi$ CM-E2 compared to M $\phi$ CM ( $5.26 \pm 0.76$  pg/ml;  $P < 0.01$ ) (Figure 1C). Similarly, TGF- $\beta$  levels were significantly higher in M $\phi$ CM-E2 ( $27.29 \pm 3.48$  pg/ml) compared to M $\phi$ CM ( $13.75 \pm 3.42$  pg/ml;  $P < 0.01$ ) (Figure 1E). Furthermore,IDO activity, as quantified by the level of kynurenine, was substantially enhanced in M $\phi$ CM-E2 ( $0.076 \pm 0.01$  pg/ml) relative to M $\phi$ CM ( $0.011 \pm 0.005$  pg/ml), representing approximately a 7-fold

increase ( $P < 0.01$ ) (Figure 1D). These findings collectively indicate that E2 effectively polarizes macrophages towards an immunosuppressive profile, characterized by enhanced expression of regulatory genes and secretion of anti-inflammatory mediators.



**Figure 1.** Evaluation of macrophages and their conditioned medium following 17 $\beta$ -estradiol treatment. Macrophages (M $\phi$ ) were treated with or without 100 nM 17 $\beta$ -estradiol (E2) for 24 hours to produce conditioned medium. (A, B) Real-time PCR showed increased Egr2 (Early growth response protein) and MR (Mannose Receptor) mRNA in E2-treated macrophages vs. controls. (C-E) M $\phi$ CM-E2 had higher IL-10, TGF- $\beta$ , and IDO (Indoleamine 2,3-dioxygenase) activity than untreated M $\phi$ CM. Data are presented as mean  $\pm$  SD of three independent experiments. Statistics: one-way ANOVA with Tukey's post-hoc test. (\*\*\*  $P < 0.0001$ , \*\*  $P < 0.001$ , ns = not significant ( $P > 0.05$ )). M $\phi$ CM, Conditioned medium isolated from untreated macrophages; M $\phi$ CM-E2, Conditioned medium isolated from macrophages treated with 17 $\beta$ -estradiol (100 nM, 24 h).

### 3.2. Clinical Efficacy of Macrophage-Conditioned Medium in a Rat Model of Rheumatoid Arthritis

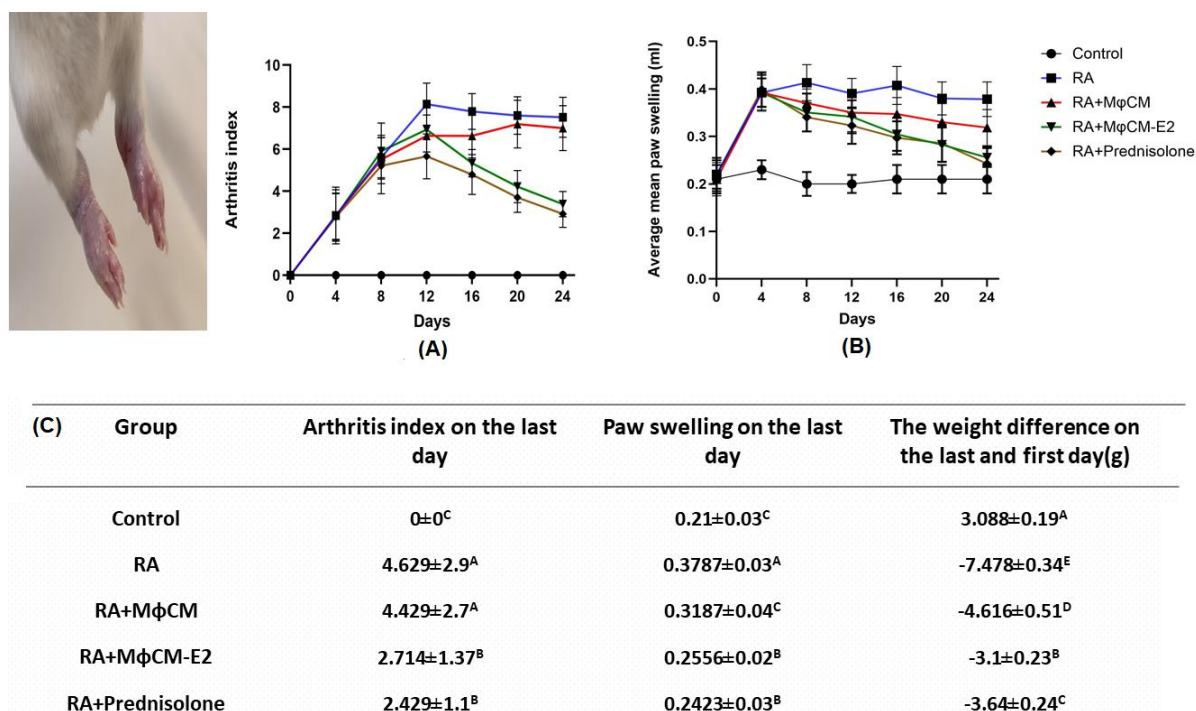
In the established Complete Freund's Adjuvant-induced arthritis model in rats, significant clinical indicators of inflammation were observed following induction (Figure 2, upper panels). Treatment commenced on day 4 post-induction when the arthritis index reached  $\geq 1$ . Untreated RA rats exhibited a progressive increase in both arthritis index and paw swelling from day 4 onwards, peaking around day 12-16 (Figure 2, graphs). On the last day of evaluation (Day 24), the untreated RA group displayed a mean arthritis index of  $4.629 \pm 2.9$  and an average mean paw swelling of  $0.3787 \pm 0.03$  mm.

Treatment with M $\phi$ CM-E2 and prednisolone demonstrated effective and statistically similar reductions in several key clinical indicators of rheumatoid arthritis in rats (Figure 2, table). On the last day, the M $\phi$ CM-E2 group showed a mean arthritis index of  $2.714 \pm 1.37$ , representing a 41.38% reduction compared to untreated RA rats. The prednisolone group achieved a comparable arthritis index of  $2.429 \pm 1.1$ , showing a 47.47% reduction ( $P < 0.05$  for both M $\phi$ CM-E2 and prednisolone vs. RA group; non-significant (ns) between M $\phi$ CM-E2 and prednisolone). Similarly, hind paw swelling on the last day was significantly reduced in the M $\phi$ CM-E2 group to  $0.2556 \pm 0.02$  mm (a 32.51% reduction from RA group) and in the prednisolone group to  $0.2423 \pm 0.03$  mm (a 36.02% reduction from RA group) ( $P < 0.05$  for both M $\phi$ CM-E2 and prednisolone vs. RA group; ns between M $\phi$ CM-E2 and prednisolone). Conversely, treatment with M $\phi$ CM alone showed no significant impact on alleviating the severity of clinical symptoms, with an arthritis index of  $4.429 \pm 2.7$  and paw swelling of  $0.3187 \pm 0.04$  mm on the last day, remaining statistically comparable to vehicle-treated RA rats (ns vs. RA group for both parameters).

Furthermore, RA induction consistently led to notable weight loss in affected animals, with the untreated RA group experiencing an average weight difference of  $-7.478 \pm 0.34$  g between the last and first day (Figure 2, table). Encouragingly, all treated animal groups exhibited improved weight gain trajectories compared to untreated RA rats. The M $\phi$ CM-E2 group showed particularly robust improvement in weight gain, with a mean weight difference



of  $-3.1 \pm 0.23$  g, which outperformed the prednisolone group (mean weight difference of  $-3.64 \pm 0.24$  g;  $P < 0.05$  vs. prednisolone). Notably, while treatment with M $\phi$ CM did not demonstrate significant improvement in clinical symptoms as previously noted, it was still effective in improving weight gain in affected rats, with a mean weight difference of  $-4.616 \pm 0.51$  g ( $P < 0.05$  vs. untreated RA rats). This suggests a distinct systemic effect of M $\phi$ CM beyond direct anti-inflammatory action on joints.



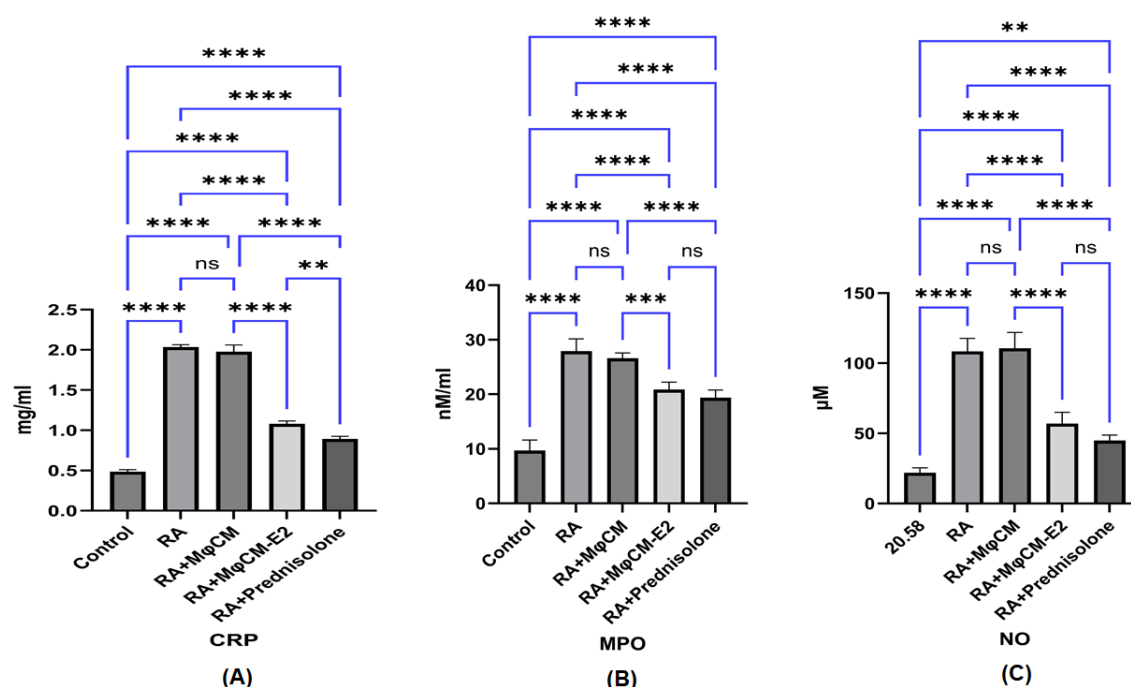
**Figure 2.** Clinical features of rheumatoid arthritis in rats following different treatments. RA was induced in rats using complete Freund's adjuvant, and treatment started 4 days post-induction until day 24. (A, B) Arthritis index and paw swelling over time. (C) Summary table. M $\phi$ CM-E2 significantly reduced the arthritis index (~41%) and paw swelling (~32%) versus untreated RA rats, similar to prednisolone. M $\phi$ CM-E2 significantly improved weight gain more than untreated RA rats and prednisolone. M $\phi$ CM alone improved weight gain but not clinical scores. Data are mean  $\pm$  SD. Statistics: one-way ANOVA with Tukey's post-hoc test. Letters (A, B, C, D, E) on the graphs indicate significant differences between the groups ( $P < 0.05$ ). M $\phi$ CM, Untreated macrophage conditioned medium; M $\phi$ CM-E2, 17 $\beta$ -estradiol (100 nM, 24 h) treated macrophage conditioned medium.

### 3.3. Biochemical Markers of Inflammation and Oxidative Stress

Biochemical analysis of serum samples from the CFA-induced arthritis model revealed a significant elevation in systemic inflammatory and oxidative stress markers in untreated RA rats compared to healthy controls (Figure 3A, 3B and 3C). CRP levels were markedly increased in RA rats ( $2.03 \pm 0.04$  mg/ml) compared to control ( $0.50 \pm 0.03$  mg/ml;  $P < 0.00001$ ). Similarly, MPO levels rose significantly in RA rats ( $27.87 \pm 2.01$  nM/ml vs.  $9.87 \pm 0.77$  nM/ml in control;  $P < 0.00001$ ), and NO levels were substantially augmented ( $107.03 \pm 6.75$   $\mu$ M vs.  $20.58 \pm 2.59$   $\mu$ M in control;  $P < 0.00001$ ).

Treatment with both M $\phi$ CM-E2 and prednisolone effectively mitigated these elevations in RA rats. Specifically, M $\phi$ CM-E2 treatment resulted in a significant reduction of CRP levels to  $1.09 \pm 0.05$  mg/ml ( $P < 0.00001$  vs. RA), while prednisolone reduced CRP to  $0.90 \pm 0.05$  mg/ml ( $P < 0.00001$  vs. RA). Prednisolone demonstrated a significantly stronger effect in reducing CRP levels compared to M $\phi$ CM-E2 ( $P < 0.001$ ). Notably, M $\phi$ CM-E2 treatment achieved a greater reduction in MPO levels, bringing them down to  $20.27 \pm 0.78$  nM/ml ( $P < 0.00001$  vs. RA group, and ns vs. prednisolone group ( $19.49 \pm 0.76$  nM/ml)). In contrast, prednisolone led to a more pronounced reduction in NO levels ( $45.33 \pm 3.49$   $\mu$ M;  $P < 0.00001$  vs. RA) compared to M $\phi$ CM-E2 ( $55.53 \pm 2.76$

$\mu\text{M}$ ;  $P < 0.00001$  vs. RA, but ns vs. prednisolone) (Figure 3A, 3B and 3C). The use of M $\phi$ CM alone did not yield a statistically significant effect on reducing serum levels of CRP ( $1.98 \pm 0.03$  mg/ml), MPO ( $26.69 \pm 1.13$  nM/ml), or NO ( $108.97 \pm 3.20$   $\mu\text{M}$ ) compared to untreated RA rats (ns for all parameters).



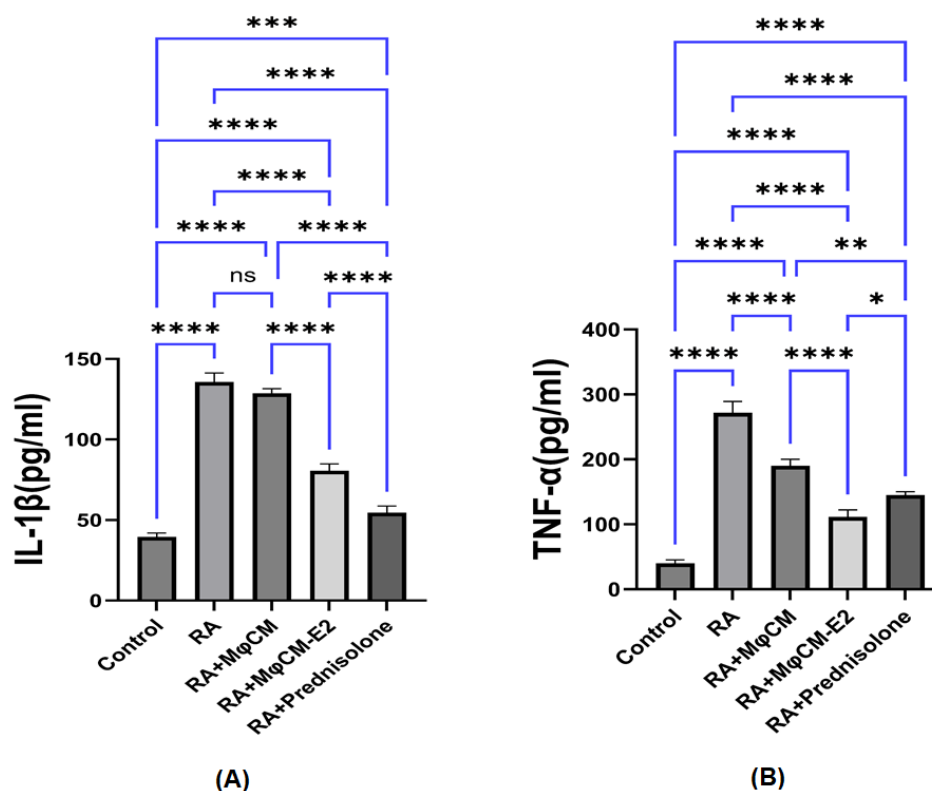
**Figure 3.** Assessment of biochemical factors in the sera of RA rats following treatment. (A) to (C) Bar graphs displaying the serum concentrations of C-reactive protein (CRP), Myeloperoxidase (MPO), and Nitric Oxide (NO). Untreated RA rats showed significantly elevated levels of these markers compared to healthy controls. Both M $\phi$ CM-E2 and prednisolone treatments significantly lowered these levels in RA rats. Prednisolone significantly reduced CRP compared to other treatment groups. M $\phi$ CM alone had no significant effect on these factors. Values are mean  $\pm$  SD. Statistics: one-way ANOVA with Tukey's post-hoc test. (\*\*\*\*  $P < 0.00001$ , \*\*  $P < 0.001$ , ns: non-significant ( $P > 0.05$ )). M $\phi$ CM, Untreated macrophage conditioned medium; M $\phi$ CM-E2, 17 $\beta$ -estradiol (100 nM, 24 h) treated macrophage conditioned medium.

### 3.4. Impact on Pro-inflammatory Cytokines

As illustrated in Figure 4, treatment with M $\phi$ CM-E2 and prednisolone led to significant decreases in the serum levels of key pro-inflammatory cytokines, IL-1 $\beta$  and TNF- $\alpha$ , in CFA-challenged rats. Serum IL-1 $\beta$  levels, which were significantly elevated in untreated RA rats ( $135.22 \pm 5.86$  pg/ml vs.  $40.89 \pm 2.96$  pg/ml in healthy controls,  $P < 0.00001$ ), were significantly reduced by both M $\phi$ CM-E2 ( $82.12 \pm 7.97$  pg/ml;  $P < 0.00001$  vs. RA) and prednisolone ( $53.38 \pm 4.54$  pg/ml;  $P < 0.00001$  vs. RA). Data analysis revealed that prednisolone resulted in a more pronounced reduction in serum IL-1 $\beta$  levels than M $\phi$ CM-E2 ( $P < 0.00001$ ). Treatment with M $\phi$ CM alone ( $129.83 \pm 6.07$  pg/ml) showed no significant effect on IL-1 $\beta$  levels compared to untreated RA (ns) (Figure 4A).

Similarly, serum TNF- $\alpha$  levels, significantly elevated in untreated RA rats ( $273.84 \pm 19.34$  pg/ml vs.  $39.56 \pm 2.93$  pg/ml in healthy controls,  $P < 0.00001$ ), exhibited the most substantial reduction in the RA group treated with M $\phi$ CM-E2, decreasing to  $112.98 \pm 12.08$  pg/ml ( $P < 0.00001$  vs. RA). This reduction by M $\phi$ CM-E2 was significantly greater than that achieved by prednisolone (reduced to  $143.76 \pm 12.63$  pg/ml;  $P < 0.001$  vs. M $\phi$ CM-E2). Treatment with M $\phi$ CM alone also lowered TNF- $\alpha$  levels to  $192.56 \pm 21.08$  pg/ml ( $P < 0.00001$  vs. RA), although the extent of this reduction was statistically less significant compared to both M $\phi$ CM-E2 ( $P < 0.00001$  vs. M $\phi$ CM) and prednisolone ( $P < 0.001$  vs. M $\phi$ CM) groups (Figure 4B).



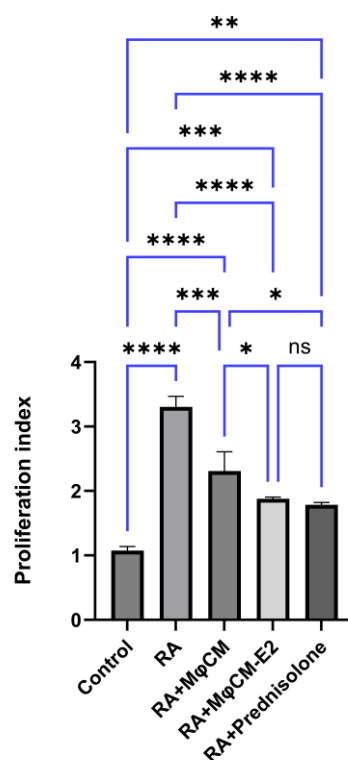


**Figure 4.** Serum levels of inflammatory Cytokines in RA Rats After Treatment. (A) and (B) Bar graphs showing the serum concentrations of IL-1 $\beta$  and TNF- $\alpha$ , respectively. In RA rats, M $\phi$ CM-E2 and prednisolone significantly lowered serum IL-1 $\beta$  and TNF- $\alpha$ . M $\phi$ CM-E2 reduced TNF- $\alpha$  more than prednisolone, while prednisolone reduced IL-1 $\beta$  more than M $\phi$ CM-E2. M $\phi$ CM lowered TNF- $\alpha$ , but to a lesser extent. Values are expressed as mean  $\pm$  S.D. Statistics: one-way ANOVA with Tukey's post-hoc test. (\*\*\*\*  $P < 0.0001$ , \*\*\*  $P < 0.0001$ , \*\*  $P < 0.001$ , \*  $P < 0.05$ , ns: non-significant ( $P > 0.05$ )). M $\phi$ CM, Untreated macrophage conditioned medium; M $\phi$ CM-E2, 17 $\beta$ -estradiol (100 nM, 24 h) treated macrophage conditioned medium.

### 3.5. Modulation of Antigen-Specific Splenocyte Proliferation

The intensity of antigen-specific splenocyte proliferation, a measure of adaptive immune response, was significantly increased in CFA-challenged rats compared to healthy controls (Figure 5). The untreated RA group exhibited a mean proliferation index of  $3.22 \pm 0.17$ , which was significantly higher than the control group ( $1.08 \pm 0.05$ ;  $P < 0.00001$ ).

Encouragingly, all therapeutic interventions resulted in a notable reduction in the splenocyte proliferation index compared to untreated RA rats. Specifically, groups treated with M $\phi$ CM-E2 (proliferation index of  $1.89 \pm 0.07$ ) and prednisolone (proliferation index of  $1.83 \pm 0.04$ ) were statistically equally effective in decreasing the lymphocyte proliferation index ( $P < 0.00001$  vs. RA for both, and ns between them). RA rats treated with M $\phi$ CM showed a significant reduction in proliferation index to  $2.29 \pm 0.28$  ( $P < 0.0001$  vs. RA), but this effect was statistically less effective than both M $\phi$ CM-E2 ( $P < 0.05$  vs. M $\phi$ CM) and prednisolone groups ( $P < 0.05$  vs. M $\phi$ CM) (Figure 5).



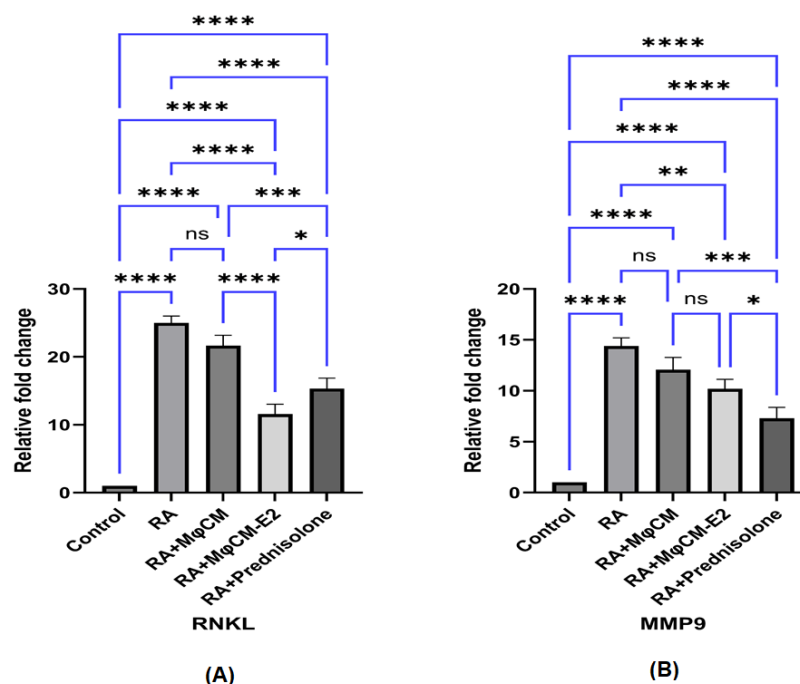
**Figure 5.** Evaluation of antigen-specific splenocyte proliferation in treated RA rats. The proliferation index of antigen-specific splenocytes was assessed in response to treatment. Untreated RA rats exhibited a significantly higher proliferation index compared to healthy controls. Both MφCM-E2 and prednisolone similarly reduced lymphocyte proliferation. RA rats treated with MφCM showed a less effective reduction compared to MφCM-E2 and prednisolone. Results are expressed as mean  $\pm$  SD. Statistics: one-way ANOVA with Tukey's post-hoc test. (\*\*\*\*  $P < 0.00001$ , \*\*\*  $P < 0.0001$ , \*\*  $P < 0.001$ , \*  $P < 0.05$ ). MφCM, Untreated macrophage conditioned medium; MφCM-E2, 17 $\beta$ -estradiol (100 nM, 24 h) treated macrophage conditioned medium.

### 3.6. Impact on Cartilage and Bone Degradation Markers in Ankle Joint Tissue

As depicted in Figure, the mRNA expression of key enzymes involved in cartilage and bone degradation, RANKL and MMP-9, exhibited significant reductions in the ankle joint tissue of rats with arthritis that received MφCM-E2 and prednisolone, compared to untreated RA rats.

In the RA group, RANKL mRNA relative fold change was  $25.04 \pm 1.05$ , significantly higher than control ( $1.00 \pm 0.00$ ;  $P < 0.00001$ ). Statistically, the effect of MφCM-E2 treatment was more pronounced in reducing mRNA expression of RANKL in the ankle joint tissue (Relative fold change:  $11.08 \pm 0.69$ ;  $P < 0.00001$  vs. RA) compared to prednisolone treatment (Relative fold change:  $15.24 \pm 1.03$ ;  $P < 0.00001$  vs. RA, but  $P < 0.05$  vs. MφCM-E2) (Figure 6A).

Conversely, the opposite effect was noted for mRNA expression of MMP-9. In the RA group, MMP-9 mRNA relative fold change was  $14.59 \pm 0.81$ , significantly higher than control ( $1.00 \pm 0.00$ ;  $P < 0.00001$ ). Here, prednisolone induced a greater reduction (Relative fold change:  $6.93 \pm 0.69$ ;  $P < 0.00001$  vs. RA) than MφCM-E2 (Relative fold change:  $10.25 \pm 0.70$ ;  $P < 0.001$  vs. RA, but  $P < 0.05$  vs. prednisolone) (Figure 6B). Ultimately, treatment with MφCM alone in rats with arthritis did not yield statistically significant reductions in any of these mRNAs in the ankle joint tissue (RANKL relative fold change:  $21.65 \pm 1.02$ ; MMP-9 relative fold change:  $12.09 \pm 0.79$ ; ns for both vs. untreated RA rats) (Figure 6A and 6B).



**Figure 6.** Determination of mRNA expression of RANKL and MMP-9 in ankle joint tissue of RA rats. (A) and (B) Bar graphs showing the mRNA expression of RANKL (Receptor Activator of Nuclear Factor Kappa-B Ligand) and MMP-9 (Matrix Metalloproteinase-9), respectively. In arthritic rats, MφCM-E2 and prednisolone significantly reduced RANKL and MMP-9 mRNA expression compared to untreated rats. MφCM-E2 reduced RANKL mRNA more than prednisolone, but prednisolone reduced MMP-9 mRNA more than MφCM-E2. MφCM alone did not significantly reduce these mRNAs. Findings are expressed as mean  $\pm$  S.D. Statistics: one-way ANOVA with Tukey's post-hoc test. (\*\*\*\*  $P < 0.00001$ , \*\*\*  $P < 0.0001$ , \*\*  $P < 0.001$ , \*  $P < 0.05$ , ns: non-significant ( $P > 0.05$ )). MφCM, Untreated macrophage conditioned medium; MφCM-E2,  $17\beta$ -estradiol (100 nM, 24 h) treated macrophage conditioned medium.

#### 4. Discussion

Immunotherapy, particularly using cell therapy, offers a more targeted approach compared to traditional immunosuppressive therapies, potentially leading to fewer side effects and improved efficacy. It aims to modulate rather than completely suppress the immune system, allowing for better management of autoimmune diseases.<sup>31,32</sup> However, cell therapy often necessitates personalized treatment for each patient. This approach involves the use of a clean room and the re-transplantation of cells into the host, making it a multi-step and costly process. The production of a conditioned medium rich in anti-inflammatory mediators from macrophages following estrogen pulsing and lyophilization, under conditions akin to those of the present study, could offer a commercializable, low-risk, low-cost, and effective solution for alleviating the symptoms of inflammatory autoimmune diseases, including RA. Lyophilization of conditioned medium for immunotherapy serves to improve stability, shelf-life, and convenience, all while preserving the biological activity of important components, ultimately supporting more effective and practical use in clinical and research contexts.<sup>33</sup>

Previous literature indicates that estradiol promotes M2/anti-inflammatory macrophage formation through a combination of receptor-mediated signaling, modulation of cytokine production, transcription factor activation (like STAT3 and p38 MAPK), changes in metabolic processes, and potentially through the regulation of microRNAs (like downregulating miR-155) and intercellular communication.<sup>34,35</sup> Estrogen boosts macrophage to produce of indoleamine 2,3-dioxygenase (IDO), an enzyme that metabolizes tryptophan, a key component in immune regulation. Increased IDO activity reduces inflammation and fosters immune tolerance by depleting tryptophan necessary for T-cell growth. IDO production is a hallmark of M2/anti-inflammatory macrophages.<sup>36</sup>

Additionally, studies indicate that estradiol increases the production of other M2 markers, such as TGF- $\beta$ , IL-10, and arginase.<sup>22</sup> NF- $\kappa$ B and STAT3 modulate macrophage M2 polarization.<sup>37-39</sup> The E2/ER $\alpha$  complex activates STAT3 and suppresses NF- $\kappa$ B, boosting IL-10 production and M2 polarization in macrophages.<sup>40,41</sup> In this context, our current study revealed a notable increase in the levels of TGF- $\beta$ , IL-10, and IDO activity in the conditioned medium obtained from E2-treated M $\phi$  compared to that from untreated M $\phi$ .

Egr2 and MR (CD206) are crucial for the induction and maintenance of anti-inflammatory M2 macrophage polarization. High levels of Egr2 and MR enable macrophages to maintain their plasticity, allowing them to adapt and respond effectively to changes in the inflammatory environment. Conversely, low levels of Egr2 and MR are linked to non-responsiveness, which can impair the immune response.<sup>42,43</sup> Our results showed that E2 treatment increased the mRNA expression of Egr2 and MR in macrophages compared to untreated ones. Overall, the results of the analysis of the conditioned medium and the RT-PCR findings suggest that treating macrophages with E2 effectively induced an anti-inflammatory phenotype similar to the M2 macrophage phenotype.

The animal studies phase of the current research indicated that while the use of M $\phi$ CM did not significantly alleviate the severity of clinical symptoms in RA rats, treatment with M $\phi$ CM-E2 and prednisolone effectively and comparably reduced clinical indices, such as the arthritis index and hind paw swelling in RA rats, thereby diminishing their suffering. The beneficial results of cell therapy using E2-treated mesenchymal stem cells (MSCs) have been noted in two recent scientific sources. A previous study revealed that exposing MSCs to E2 at a concentration of 100 nM for 24 hours significantly enhanced the suppression of T lymphocyte proliferation, increased IDO, IL-10, nitric oxide, and TGF- $\beta$  production, and upregulated CXCR4 and CCR2 mRNA expression in MSCs. This treatment showed greater efficacy in alleviating RA severity in rats compared to MSCs alone.<sup>30</sup> In another experiment, it was observed that treatment with E2-pulsed MSCs led to a reduction in the cumulative clinical score and a significant enhancement in neuropathology in rats with an experimental form of multiple sclerosis, compared to treatment with un-pulsed MSCs.<sup>44</sup>

Myeloperoxidase (MPO), an enzyme produced by neutrophils, plays a crucial role in oxidative stress and inflammation, serving as a biomarker for various conditions, including RA. Elevated serum MPO levels in RA patients correlate with increased disease activity and other inflammatory markers, indicating its potential for evaluating disease progression and treatment efficacy.<sup>45,46</sup> Similarly, the serum level of nitric oxide (NO) is significantly higher in RA patients and correlates with disease severity.<sup>47,48</sup> The data obtained from the current survey indicated that, in contrast to treatment with M $\phi$ CM, which did not affect MPO and NO levels, M $\phi$ CM-E2 demonstrated greater effectiveness than prednisolone in reducing both MPO and NO levels. Additionally, C-reactive protein (CRP), an inflammatory marker produced in response to cytokines, is also elevated in RA and correlates with disease activity;<sup>49,50</sup> however, M $\phi$ CM-E2 treatment effectively lowers CRP levels, unlike M $\phi$ CM. Collectively, these findings highlight the significance of MPO, NO, and CRP as biomarkers in monitoring RA and the potential of M $\phi$ CM-E2 treatments in managing inflammation. Recent experiments indicate that E2-pulsed MSCs have a similar efficacy to prednisolone in reducing RA symptoms and lowering levels of CRP, RF, and NO.<sup>30</sup> Additionally, a notable decrease in serum levels of NO and MPO was found in rats with experimental autoimmune encephalomyelitis (EAE) treated with E2-pulsed MSCs compared to those receiving untreated MSCs.<sup>44</sup>

IL-1 and TNF- $\alpha$  significantly promote inflammation in joint synovial tissue, recruiting more immune cells and perpetuating joint damage.<sup>51</sup> Previous studies showed that MSC-conditioned media have anti-inflammatory

effects by inhibiting the NF- $\kappa$ B and MAPK pathways, resulting in reduced activation of inflammatory mediators like IL-1 and TNF- $\alpha$ , thus promoting a balanced immune response.<sup>52</sup> Here, we reported that treatment with M $\phi$ CM-E2 significantly decreased serum IL-1 $\beta$  and TNF- $\alpha$  levels in CFA-challenged rats, whereas treatment with M $\phi$ CM was effective only in reducing TNF- $\alpha$  levels.

RANKL is a pivotal mediator in the pathophysiology of RA-related bone erosion through its role in promoting osteoclast differentiation and activity. RANKL expression is influenced by various inflammatory cytokines such as IL-1, IL-6 and TNF- $\alpha$ , which are upregulated in RA.<sup>53</sup> MMP-9 exhibits a dual role in RA progression, promoting both cartilage degradation and enhanced inflammatory responses within rat ankle joints. MMP-9 expression correlates with disease severity in RA.<sup>54</sup> In previous studies, mainly based on conditioned medium obtained from MSCs, it has been shown that MSC-conditioned medium significantly impacts RANKL and MMP-9 levels, thereby influencing osteoclast differentiation and activity.<sup>55,56</sup> According to our results, unlike M $\phi$ CM, treatment with M $\phi$ CM-E2 significantly decreased RANKL and MMP-9 mRNA expression in ankle joint tissue compared to untreated rats.

## 5. Conclusion

In comparison to conditioned medium isolated from M $\phi$ , pretreatment of M $\phi$  with estradiol promotes a potent anti-inflammatory milieu in the secretome of M $\phi$ . An in vivo study indicated that M $\phi$ CM-E2 caused more profound beneficial effects in the treatment of RA in Wistar rats compared to RA rats treated with M $\phi$ CM. The outcomes of treatment with M $\phi$ CM-E2 were comparable to those of prednisolone. Given the excellent potential of M $\phi$ CM-E2, this approach may represent a promising and beneficial strategy for RA immunotherapy. However, this survey is a preliminary study in an animal model, and further studies are required to demonstrate the efficacy of M $\phi$ CM-E2 in humans with RA.

## Authors Contributions

The research design was by SMAF and AO. The experiments were conducted by MP. The data analysis and preparation of the final manuscript were done by SMAF. All authors have read the final manuscript and declare no conflicts of interest.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests.

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## Ethical approval

Ethical considerations concerning the use of laboratory animals were conducted in accordance with the regulations set forth by the Iranian Ministry of Health and the Helsinki Convention, following approval from the Ethics Committee of the Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

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