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Original Research

Long term treatment by mesenchymal stem cells conditioned medium modulates cellular, molecular and behavioral aspects of adjuvant-induced arthritis

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Abstract: Neuroinflammation plays a crucial role in expression of symptoms of numerous autoimmune and neurodegenerative diseases such as pain during rheumatoid arthritis. Overproduction of pro-inflammatory cytokines and activation of intracellular signaling pathways have been strongly implicated in the generation of pathological pain states, particularly at central nervous system sites and induction of spinal neuroinflammatory symptoms. The wide ranges of research to define new therapeutic approaches, including neuroimmune-modulators like stem cells are in progress. Mesenchymal stem cells conditioned medium (MSC-CM) has anti-inflammatory factors which can regulate the immune responses. The aim of this study was to investigate the effect of administration of MSC-CM on behavioral, cellular and molecular aspects of adjuvant-induced arthritis in male Wistar rats. Complete Freund's adjuvant (CFA)-induced arthritis (AA) was caused by single subcutaneous injection of CFA into the rat's hind paw on day 0. MSC-CM was administered daily (i.p.) and during the 21 days of the study after injection. Hyperalgesia, Edema, Serum TNF-α levels and p38MAPK and NF-κB activities were assessed on days 0,7,14 and 21 of the study. The results of this study indicated the role of MSC-CM in reducing inflammatory symptoms, serum TNF-α levels and activity of intracellular signaling pathway factors during different phases of inflammation caused by CFA. It seems that MSC-CM treatment due to its direct effects on inhibition of intracellular signaling pathways and pro-inflammatory cytokines can alleviate inflammatory symptoms and pain during CFA-induced arthritis.

Key words: Neuroinflammation; Rheumatoid arthritis; MSC-CM; Hyperalgesia; Edema; TNF-α; P38MAPK; NF-κB.

Introduction

Nowadays, a considerable body of work has suggested that peripheral inflammation and central neuroimmune cell activation lead to production of inflammatory cytokines, and expression of surface antigens which can spark-off central nervous system (CNS) immunological cascades (1). So, numerous pain mediators which can sensitize and lower the threshold of neuronal firing, increase responses to suprathreshold stimuli, and stimulate ongoing spontaneous activity in the dorsal horn of the spinal cord have been produced. Consequently, spinal neuroinflammation predominantly incur central sensitization, which refers to an enhanced responsiveness of nociceptors in the CNS to afferent inputs (2).

Pro-inflammatory cytokines are pivotal mediators of neuroinflammation which can cause sensitization, and neuropeptides secreted from nociceptors can, in turn, accentuate the inflammatory responses and orchestrated actions of immune cells. These findings proposed that alterations which are caused by central sensitization are manifested in both behavioral and spinal neuroinflammatory responses (1, 2). Moreover, constitutive overproduction of spinal pro-inflammatory cytokines, including tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) in damaged cells and areas, has been implicated to play a pathologic role in the neuroinflammation, chronic autoimmune and neurodegenerative disorders pathogenesis such as Rheumatoid Arthritis (RA). RA is a severe chronic inflammatory autoimmune disease which is characterized by progressive cartilage destruction, hyperalgesia and edema (3, 4). Increased levels of serum TNF-α play an extremely central role in driving spinal neuroinflammation, hyperplasia and aggregation of leukocytes in the area (5). Several studies have demonstrated roles for P38MAPK (P38 mitogen-activated protein kinases) in microglia and astrocyte driven spinal neuroinflammation. P38MAPK activation followed by the expression of NF-κB (Nuclear factor κ-light-chain-enhancer of activated B cells) participates in the production of cytokines and chemokines and also plays a pivotal role in the development of hyperalgesia and spinal neuroinflammatory symptoms (6, 7). Studies have shown that inhibition of pro-inflammatory cytokines and intracellular signaling pathways are effective in reducing the neuroinflammatory symptoms such as pain, bone and cartilage destruction in RA (4, 8).

One area that has emerged as a promising therapeutic objective for the treatment of RA and alleviation of spinal neuroinflammatory symptoms is the modulation of CNS immunological responses. The uses of synthetic drugs such as corticosteroids, which are commonly prescribed for the treatment of inflammatory diseases, are associated with many downsides. So, the wide ranges of research to define new therapeutic approaches, including neuroimmune-modulators like stem cells are

in progress (9). Possible mechanisms of the therapeutic effects of these cells are due to their neuroimmune and immuno-modulatory valences and paracrine effects as well (9, 10).

Conditioned medium (CM) is a supernatant that is extracted from the grown stem cells which contains growth factors, chemokines and extracellular matrix proteins (10, 11). MSC-CM (mesenchymal stem cells conditioned medium) has immune regulatory properties and can modify the functions (proliferation, activation and effective performance) of a wide variety of immune cells including B and T lymphocytes (9, 10). Whereas MSC-CM has been hypothesized as having a role in inflammatory symptoms alleviation due to its trophic factors, but its physiological manifestations, roles in behavioral, central and peripheral aspects of inflammatory arthritis and modulation of central neuroinflammation have not been demonstrated and further investigations are needed. Then, based on the important role of the cellular and humoral immunity in the induction of neuroinflammatory symptoms of arthritis and potency of MSC-CM in modulation of immune responses, in this study, the effect of long term treatment with MSC-CM on peripheral and central, cellular, molecular and behavioral aspects of inflammatory pain during different stages of adjuvant-induced arthritis was investigated.

Materials and Methods

Laboratory animals

In this study, the adult male Wistar rats (n=120) weighing 200–220 g were randomly selected. These rats were housed in polypropylene cages under hygienic and standard environmental circumstances (22±2ºC, humidity 60–70 %, 12 h light/dark cycle). All the procedures were approved by the guidelines of the ethical standards for the investigations of experimental pain in animals (12). In order to determine the effect of MSC-CM on arthritis model and the effectiveness of this treatment, a series of experiments were executed. Rats were randomly divided into 5 experimental groups, as follows: (a) CFA group, (b) CFA control group, (c) CFA+CM, (d) CFA+FBS (Fetal Bovine Serum as vehicle of MSC-CM), (e) Sham group. According to the study procedure, each group was divided into four subgroups based on different time points of the study (days 0, 7, 14, and 21) (n=6/subgroup).

CFA arthritis induction and assessment of paw edema

CFA-induced arthritis was caused by single subcutaneous injection of (100µL) heat-killed Mycobacterium tuberculosis suspended in sterile mineral oil (10 mg/ml; CFA; Sigma, St Louis, MO, USA) into the rats' right hind paw on day 0 (under light anesthesia).

To confirm the correct measurement of CFA injection, the volume of both the injected and the contralateral paws before and after the injection was tested with plethysmometer apparatus during different time points of the study (model 7141; Ugo Basile, Comerio-Varese, Italy) (13). In brief, the rat hind paw was submerged to the Tibiotarsal joint into a transparent chamber containing an electrolyte solution. The volume of the displacement, which is equal to the paw volume, was indicated on a digital display. For each paw, a measurement was done twice and the average was calculated. The edema was quantified by measuring the differences in the foot volume between the day 0 and other different time points of the study (14).

Behavioral test (thermal hyperalgesia assessment)

Paw withdrawal latencies (PWL) in response to radiant heat by plantar test was performed in the control and experimental groups (Ugo Basilar, Verse, Italy). 15 minutes before the test, rats were placed in a Plexiglas chambers in order to habituate to the test environment. Infrared light was projected into the hind paw focally. A cut off time of 20 seconds was considered to avoid paw injury. PWL, for each paw at an interval of 5 - 10 min, was done and the mean latency of the withdrawal responses for each paw was calculated. The calculated amount of the injected paw was subtracted from the other paw and the value obtained in the negative represented the hyperalgesia in the injured paw (15).

Blood sampling and serum TNF-α measurements

Rats were anesthetized moderately with methoxy isoflurane and retro-orbitally blood samples were prepared and collected in heparinized tubes and centrifuged (in 4ºC and 13000rpm). Serum TNF-α levels were evaluated by enzyme-linked immunosorbent assay (ELISA) kit (Bender MED System, UK) according to the manufacturer's instruction. The procedure summarized as follow: Blood serum was added to 96-well micro plates coated with rat TNF-α-specific polyclonal antibody. After the incubation at room temperature for 2 hours, followed by washing, enzyme-linked polyclonal antibody specific for TNF-α was then added. After incubation for 2 hours at room temperature and washing again, the color reagents were added. The color intensity was measured by Microplate reader at 450 nm. A standard curve was made and the TNF-α levels (pg/ml) of the samples were calculated (3, 13).

Spinal cord tissue extraction for western blotting

In order to western blotting and identifying the activation of spinal P38MAPK and NF-κB in each phase of the study, rats were anesthetized with methoxy isoflurane and their heads removed. Their lumbar segment of the spinal cord (L1–L5) separated rapidly on ice, and then they froze in liquid nitrogen at a temperature of -80 °C were maintained (6).

P38MAPK, Pp38MAPK and NF-κB Expression detection by western blotting

Samples in Lysis buffer containing proteinase inhibitors and phosphatase inhibitors were homogenized, centrifuged (45 min, 13000rpm, 4ºC) and the supernatant was collected. Then the diluted proteins with loading buffer were loaded in each lane on 12.5 % sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and run at 100mv for 2 hours. Separated proteins were transferred to Immobilon-PVDF membranes (Millipore, Bedford, MA, USA) using the miniprotean II (Bio-Rad). Nonspecific binding sites on the membrane were blocked by incubation (90 min at 24ºC) in blocking buffer. After that the membrane was incubated with primary antibodies in blocking buffer (Anti-Pp38

antibody, ab31828; Anti-p38 antibody, ab32557; anti-NF-κB p65 antibody, ab16502; and Anti-beta actin antibody, ab119716) and secondary antibodies (Anti-rabbit and anti-mouse antibodies) in blocking buffer (Abcam, CA). The immunoreactivity of the proteins on the membrane was visualized by a chemiluminescence detection system (ECL, Amersham). Band intensities were measured densitometrically by means of J-Image software. It should be noted that to measure the variation of spinal P38MAPK and NF-κB expression, the ratio of Pp38 to total P38MAPK and NF-κB to beta actin were calculated respectively (3, 6).

MSC-CM preparation, characterization and administration

Bone marrows of femurs and tibias of two-month-old male Wistar rats were obtained (200-250 g), soaked in cold PBS and removed adherent soft tissues. Bone marrow (contains hematopoietic stem cells and stromal cells) was cultured in minimal essential medium alpha (α-MEM, Gibco, Invitrogen, Carlsbad, CA, USA) containing 15% FBS (Gibco, Invitrogen, Carlsbad, CA, USA) and 1% Penicillin/streptomycin (Gibco, Invitrogen, Carlsbad, CA, USA) and incubated at 37°C in the presence of 5% carbon dioxide. The cells reach to passages three were used for following studies. Flowcytometry analysis was used for approving of MSCs and their multipotent property. After 48 hours, the medium of cells was replaced. After 48 hours, the medium in all flasks (MSCs supernatant) were collected, mixed and filtered by the 0.2 micrometers. Experimental groups received the MSC-CM on a daily basis (250µL/rat) (i.p.) till 21 days of the study at the same time (16).

Flowcytometry analysis for characterization of MSCs

Cultured MSCs were detached by Trypsin/EDTA and washed 2 times with PBS. About 2×10^5 of cells were incubated with an appropriate concentration of FITC-conjugated monoclonal rat anti-mouse CD31, CD34, CD44, CD45, CD73, CD90, CD105 and CD106 for 40 minutes at 4°C in the dark. Quantitative fluorescence analysis was done using FACS Caliber Cytometer (Becton Dickinson, San Diego, CA, USA) and Cell Quest software. At least 20000 events were collected. All the experiments also incubated with FITC- rat anti-mouse IgG1 as a negative isotype control (16).

Experimental procedure

Arthritis was caused by single subcutaneous injection of (100µL) CFA into the rats' right hind paw on day 0. The CFA control group received sterile mineral oil once (100µL, S.C.). MSC-CM group was received the MSC-CM on a daily basis (i.p., 250µL/rat) (17). The CFA+FBS group received CFA once (S.C., 100µL) and FBS on a daily basis (i.p., 250µL/rat). The sham group received sterile mineral oil on day zero (S.C., 100µL) and MSC-CM on a daily basis (i.p., 250µL/rat). The behavioral test, paw edema, serum TNF-α levels and molecular measurements (assessment of spinal P38MAPK & NF-κB), were assessed on day zero (immediately before CFA injection), on days 7 (inflammatory phase), 14 and 21 (arthritic phase) (Figure 1) (4, 15).

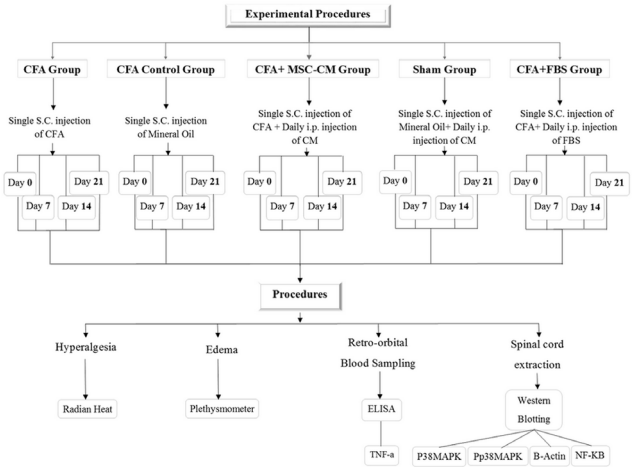

Figure 1. Experimental procedure has been shown in figure 1 entirely. Hyperalgesia, Edema, Serum levels of TNF-α and P38MAPK and NF-κB expressions were assessed on days 0,7,14 and 21 of the study in different study groups.

Statistical analysis

Results were expressed as SEM±Mean. For comparison variants within the group, repeated measurement ANOVA test (One way ANOVA) and post hoc *Tukey* were used and to compare more accurately the changes variants on the same days between the two groups, unpaired student *t*-test was used. Statistical significance was accepted at $P\leq0.05$.

Results

Isolation and characterization of cultured MSCs

The isolated MSCs demonstrated homogenous fibroblastic-like morphology in vitro, which tightly attached to the culture dish. Results of flow cytometric analysis on expression of cell surface antigens illustrated that MSCs were expressed CD44, CD73, CD90, CD105, and CD106 while were negative for CD31, CD34, and CD45 (Figure 2a, b).

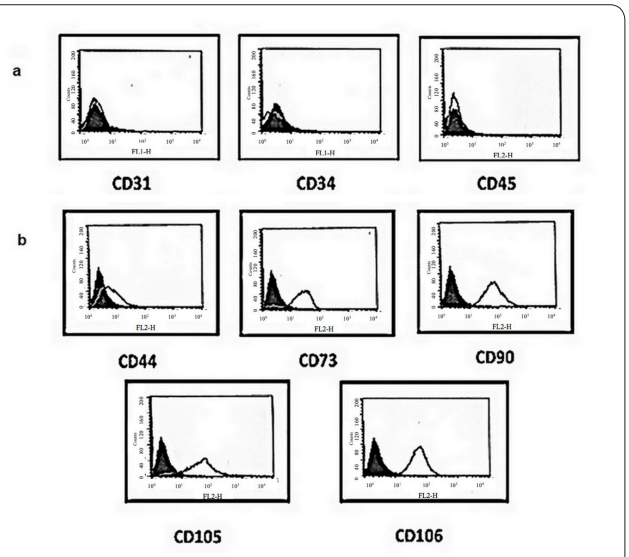

Figure 2. Characterization of rat BM-MSC surface markers by using flowcytometry. a: Flowcytometric analysis demonstrated that cultured cells were positive for CD44, CD73, CD90, CD105 and CD106. b These cells were negative for CD31, CD34, and CD45. The respective isotype control is shown as black lines. The data are representative of three independent experiments.

Paw edema variations during different stages of arthritis inflammation

CFA injection into the rat's hind paw induced inflammation and edema in the affected paw, which continued until 21 days after CFA injection. Paw volume on days 7, 14 and 21 compared with baseline in the CFA group noticeably increased (P≤0.01 for day 7 and P≤0.001 for day 14 and 21). No significant differences in paw volume was shown in the CFA Control, Sham and CFA+FBS groups compare with their baselines (Therefore, the results of the sham and CFA+FBS groups are not shown graphically) (Figure 3a). Paw edema was significantly reduced in the CFA+CM group on days 7, 14 and 21 compared with the same days in the CFA group (P≤0.05 for day 7, P≤0.01 for day 14 and P≤0.001 for day 21). Continuing injection of MSC-CM declined paw edema in the CFA+CM group, so that at day 21 of the study, no significant difference in paw edema in the CFA+CM group in comparison with the CFA Control group was observed (Figure 3b).

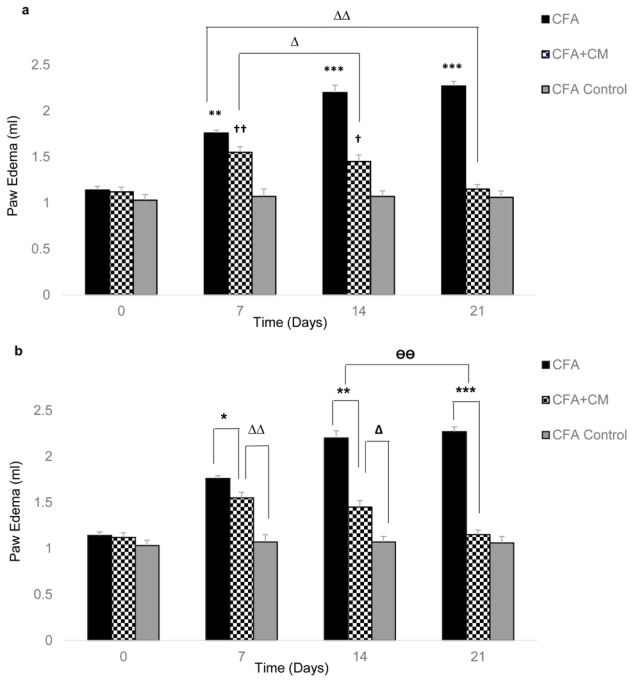

Figure 3. Paw edema significantly increased in CFA group compared with baseline in different time points of the study. Long-term administration of MSC-CM was noticeably downturned paw edema. Results as Mean±SEM (n=6/group) stated. **P≤0.01 and ***P≤0.001 for comparison of paw edema variations between baseline and different days of the study in CFA group. †P≤0.05 and ††P≤0.01 for comparison of paw edema variations between baseline and different days of the study in the CFA+CM group. ΔP≤0.05 and ΔΔP≤0.01 for indicating the changes in paw edema at days 14 and 21 compared with day 7 in CFA+CM group. **b** CFA injection considerably increased paw edema, while MSC-CM injection caused a significant reduction in paw edema compared with CFA group. Results as Mean±SEM (n=6/group) stated. *P≤0.05, **P≤0.01 and ***P≤0.001 for comparison of paw edema variations between CFA and CFA+CM groups in the same days. ΔP≤0.05 and ΔΔP≤0.01 for comparison of paw edema variations between CFA+CM and CFA Control groups in the same days. ΘΘP≤0.01 for comparison of differences in paw edema variations in CFA and CFA+CM groups at day 14 compared with day 21.

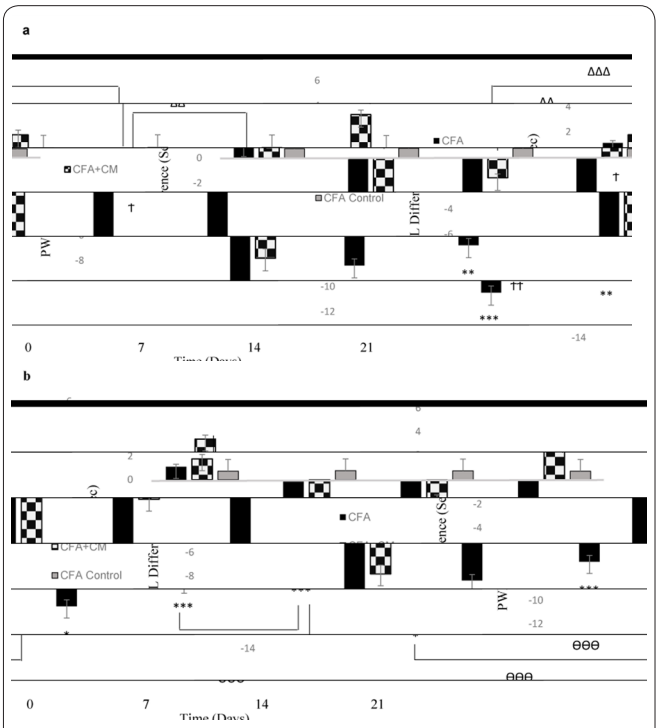

Figure 4. a. Hyperalgesia significantly increased in different time points of the study in the CFA group compared with baseline and long-term administration of MSC-CM remarkably decreased thermal hyperalgesia. Results as Mean±SEM (n=6/group) stated. **P≤0.01 and ***P≤0.001 for comparison of the paw withdrawal latency variations between baseline and different days of the study in CFA group. †P≤0.05 and ††P≤0.01 for comparison of paw edema variations between baseline and different days of the study in CFA+CM group. ΔΔP≤0.01 and ΔΔΔP≤0.001 for comparison of changes in hyperalgesia at days 14 and 21 compared with day 7 in CFA+CM group. b. CFA injection noticeably increased hyperalgesia while MSC-CM injection significantly declined hyperalgesia compared with CFA group. Results as Mean±SEM (n=6/group) stated. *P≤0.05 and ***P≤0.001 for comparison of hyperalgesia variations between CFA and CFA+CM groups in the same days. ΘΘΘP≤0.001 for comparison of differences in paw edema variations in CFA and CFA+CM groups at days 7 and 14 compared with day 21.

Thermal hyperalgesia variations during different stages of arthritis inflammation

CFA injection in the rat's hind paws induced inflammation and hyperalgesia, which continued up to day 21. Hyperalgesia significantly increased on the 7th day in the CFA group (P≤0.01), but remarkably decreased on days 14 and 21 of the study. However, there was still a significant increase compared to baseline (P≤0.01). No significant differences in PWL was observed in the CFA Control, Sham and CFA+FBS groups compare with their baselines (Therefore, the results of the sham and CFA+FBS groups are not shown graphically). Daily injection of MSC-CM significantly declined hyperalgesia in the CFA+CM group, as the continuity of this injection for 21 days also reduced hyperalgesia even more than the day zero (P≤0.05)(Figure 4a). Hyperalgesia significantly reduced in the CFA+CM group on days 7, 14 and 21 compared with the CFA group (P≤0.05 for day 7 and P≤0.001 for days 14 and 21). Significant differences in the PWL in different time points of the study between CFA and CFA Control groups were shown (P≤0.001) (Figure 4b).

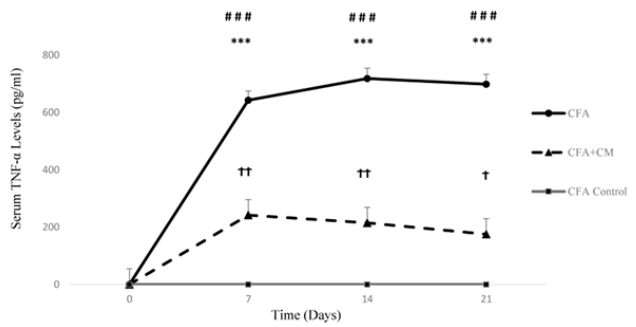

Figure 5. TNF-α levels were considerably increased in different time points of the study in the CFA group compared with baseline. Long-term administration of MSC-CM significantly decreased the serum levels of TNF-α. Results as Mean±SEM (n=6/group) stated. ***P≤0.001 for comparison of variations in serum TNF-α levels between baseline and different days of the study in CFA group. †P≤0.05 and ††P≤0.01 for comparison of variations in the serum TNF-α levels between baseline in different days of the study compared with CFA+CM group. ‡‡‡P≤0.001 for comparison of changes in serum TNF-α levels in different days of the study in CFA and CFA+CM groups.

Serum TNF-α levels variations during different stages of arthritis inflammation

Arthritis caused by CFA injection, significantly increased serum levels of TNF-α on different time points of the study in the CFA group compared with day zero (P≤0.001). No significant differences in serum levels of TNF-α was observed in the CFA Control, Sham and CFA+FBS groups compare with their baselines (Therefore, the results of the sham and CFA+FBS groups are not shown graphically). Significant differences in the serum TNF-α levels on different days between CFA and CFA Control groups were observed (P≤0.001). Serum levels of TNF-α were decreased considerably in the CFA+CM group compared with the CFA group (P≤0.001) (Figure 5).

Spinal Pp38MAPK/P38MAPK expression variations during different stages of arthritis inflammation

In order to detect spinal P38MAPK enzyme activity, Pp38MAPK monoclonal antibody was used. Protein expression of P38MAPK indicated resembling bands with molecular masses of nearly 42 kDa in the spinal cord tissues of all experimental groups. P38MAPK was considered as a housekeeping protein. Accordingly, for normalizing the differences in protein loading, all the data were represented as Pp38/P38MAPK ratios. Immuno-specificity was corroborated by the absence of immune-reactive bands when the membrane was pre-incubated with an antigenic peptide prior to antibody incubation.

Densitometry of the data which were obtained from the Western blotting technique illustrated that phosphorylation of P38MAPK enzyme in the spinal cord of rats in the CFA group significantly increased in different days of the study compared to day zero (P≤0.01 for day 7 and P≤0.001 for day 14 and 21). No significant changes in the spinal Pp38/P38MAPK activity was observed in the CFA Control, Sham and CFA+FBS groups compare with their baselines (Therefore, the results of the sham and CFA+FBS groups are not shown graphically). Spinal Pp38/P38MAPK activity noticeably decreased on days 14 and 21 in the CFA+CM group compared with the CFA group (P≤0.01 for day 14 and P≤0.001 for day 21). This reduction in day 21 was in such a way that no considerable differences in the Pp38/P38MAPK activity in the CFA+CM group compared with CFA control group was observed (Figure 6a, b).

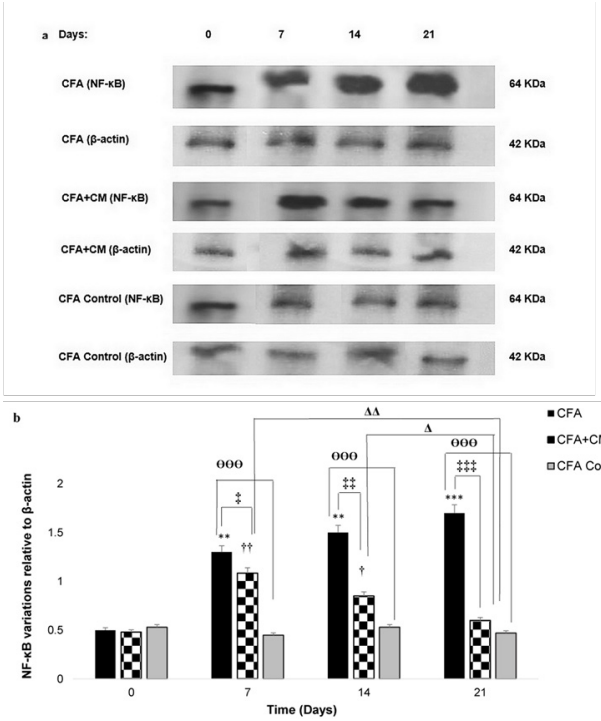

Figure 6. a. Immunoblots of spinal P38MAPK enzyme expression during different stages of inflammation (day 0, 7, 14 and 21) in CFA and CFA+ CM groups. All densitometry data were demonstrated as Pp38/P38MAPK ratio. Data are represented as Mean±SEM (n=6/group). b P38MAPK enzyme activity during different stages of inflammation in all experimental groups. The Pp38/P38MAPK ratio significantly increased in days 7, 14 and 21 after CFA injection compared with day 0 in CFA group. The Pp38/P38MAPK ratio noticeably declined at day 21 compared with day 7 in CFA+CM group. ***P≤0.001 and **P≤0.01 for comparison of spinal P38MAPK protein band intensity in different days of the study compared with baseline in the CFA group. ††P≤0.01 and †P≤0.05 for comparison of spinal P38MAPK protein band intensity in different days of the study compared with baseline in CFA+CM group. ΔΔP≤0.01 for comparison of spinal P38MAPK protein band intensity in day 21 compared to day 7 in CFA+CM group. ‡‡P≤0.01 and ‡‡‡P≤0.001 for comparison of changes in the Pp38/P38MAPK ratio in different days of the study in CFA and CFA+CM groups. ΘΘΘP≤0.001 for comparison of changes in the Pp38/P38MAPK ratio in different days of the study in CFA and CFA Control groups.

Spinal NF-κB expression variations during different stages of arthritis inflammation

In order to detect spinal NF-κB expression, anti-NF-κB p65 polyclonal antibody was used. Protein expression of NF-κB indicated resembling bands with molecular masses of nearly 64 kDa in the spinal cord tissues of all experimental groups. Beta-actin was considered as a loading control protein. Accordingly, for normalizing the differences in protein loading, all the data were represented as NF-κB/Beta-actin ratios. Immuno-specificity was corroborated by the absence of immune-reac-

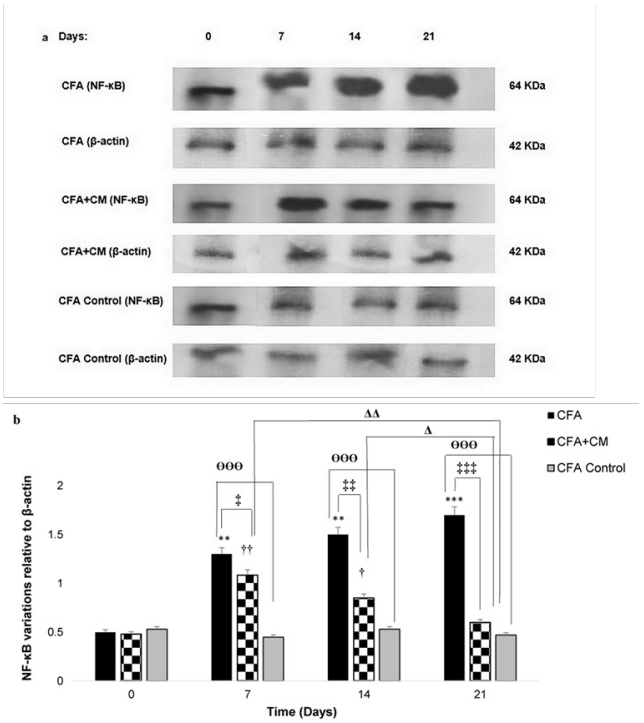

Figure 6. a. Immunoblots of spinal NF-κB expression during different stages of inflammation (day 0, 7, 14 and 21) in CFA and CFA+CM groups. All densitometry data were illustrated as NF-κB/Beta-actin ratio. Data are represented as Mean±SEM (n=6/group). b NF-κB expression during different stages of inflammation in all experimental groups. The NF-κB/Beta-actin ratio considerably elevated in days 7, 14 and 21 after CFA injection compared with day zero in CFA group. The NF-κB/Beta-actin ratio significantly decreased at day 21 compared with day 7 in CFA+CM group. ***P≤0.001 and **P≤0.01 for comparison of spinal NF-κB protein band intensity in different days of the study compared with baseline in CFA group. ††P≤0.01 and †P≤0.05 for comparison of spinal NF-κB protein band intensity in different days of the study compared with baseline in CFA+CM group. ΔP≤0.05 and ΔΔP≤0.01 for comparison of spinal NF-κB protein band intensity in day 21 compared with days 14 and 7 respectively in CFA+CM group. ‡P≤0.05, ‡‡P≤0.01 and ‡‡‡P≤0.001 for comparison of changes in NF-κB/Beta-actin ratio in different days of the study in CFA and CFA+CM groups. ΘΘΘP≤0.001 for comparison of changes in NF-κB/Beta-actin ratio in different days of the study in CFA and CFA Control groups.

tive bands when the membrane was pre-incubated with an antigenic peptide prior to antibody incubation.

Densitometry of the data which were obtained from the Western blotting technique illustrated that phosphorylation of NF-κB in the spinal cord of rats in the CFA group considerably elevated in different days of the study compared to baseline (P≤0.01 for day 7 and P≤0.001 for day 14 and 21). Significant changes in the phosphorylation of NF-κB during 21 days of the study in the CFA Control, Sham and CFA+FBS groups were not observed (Hence, graphing results were not displayed). MSC-CM can considerably reduce the NF-κB/Beta-actin expression in days 7, 14 and 21 in the CFA+CM group compare with the CFA group (P≤0.05 for day 7, P≤0.01 for day 14 and P≤0.001 for day 21). This reduction in day 21 was in such a way that no remarkable differences in the NF-κB/Beta-actin expression in the CFA+CM group compared with CFA control group was observed (Figure 7a, b).

Discussion

The present study documented that MSC-CM which was extracted from cultured MSCs that were positive for cell surface molecules including CD90, CD105, CD166, CD44, and CD29 and negative for CD34, CD14, and CD45 could reduce edema, hyperalgesia, serum levels of TNF-α and spinal P38MAPK and NF-κB expression during acute and chronic phases of CFA-induced arthritis. The continuing administration of MSC-CM reduced the inflammatory symptoms to a level of control group.

In this study, our results revealed that long term treatment by MSC-CM in CFA-injected rat's hind paw reduced hyperalgesia and edema in parallel with decreasing of serum TNF-α level during both acute and chronic stages of neuroinflammation. Our previous studies in line with the results of this study have shown that edema and hyperalgesia induced by CFA, as an animal usual model of RA, were started two hours after the injection in the rat's hind paw and continued for at least 21 days (3, 4). Hyperalgesia significantly increased on the 7th day after the CFA injection, but considerably decreased on days 14 and 21 of the study compared with day 7. Arthritis model induced by CFA is a two-phase model which is accompanied with pro-inflammatory cytokines elevation (18). Recent research in the neurobiology of cytokines has shown that pro-inflammatory cytokines as neuromodulators and potential neurotransmitters in the CNS have been strongly implicated in the generation of pathological pain states, particularly at CNS site and their pleiotropic actions can adjust the inflammatory responses of immune system cells. They can modulate amplitude and duration of the immune responses. Data from diverse animal models support the idea that TNF-α via endothelial damage and incrementing of vascular permeability can cause edema (19, 20). The results of this study also showed that arthritis caused by CFA injection significantly increased serum levels of TNF-α, ergo, neutralizing of this cytokine could decrease hyperalgesia and edema during different stages of neuroinflammation. Therefore, it seems that elevated levels of serum TNF-α within 21 days of the study may play important role in continuation of edema and changes in hyperalgesia during RA (19). Besides, studies have stated that MSC-CM injection increased expression of anti-inflammatory cytokines, decreased expression of pro-inflammatory cytokines and activity of intracellular signaling pathways and through these immune-modulatory actions can alleviate spinal neuroinflammatory symptoms (10, 21, 22). Our results not only represent anti-inflammatory and effectual role of MSC-CM in the acute phase of arthritis induced by CFA, but also showed that continuing administration of MSC-CM during this animal model of RA could reduce spinal neuroinflammatory symptoms in the chronic arthritic phase. So, these results showed that MSC-CM could exert its anti-inflammatory effects through peripheral mechanisms in RA which had not shown up to now.

On the other hand, emerging evidence suggests that TNF-α has indirect anti-inflammatory effects during the chronic phase of neuroinflammation. Akhtari et al. have shown that inhibition of serum TNF-α, can exacerbate neuroinflammatory symptoms in the chronic phase of inflammation through inhibiting the secretion of IL-10

as an anti-inflammatory cytokine (13). Hence, it seems that despite the decreased amount of serum TNF-α, anti-inflammatory effects of MSC-CM in chronic phase of arthritis were occurred by different pathways such as increasing the level of anti-inflammatory mediators and variation of cell signaling pathways. Accordingly, Tu-Lai Yew, et al. have shown that treatment with MSC-CM not only reduced the secretion of TNF-α and IFN-γ, but also could increase the secretion of anti-inflammatory cytokines in the site of inflammation (21). In this regard, our results demonstrated that active phosphorylated form of P38MAPK (Pp38MAPK) and NF-κB as a result of long-term injection of MSC-CM in the chronic phase of arthritis was reduced, which also was aligned with reduction of edema and hyperalgesia subsequently. Then decreased level of expression of MAPK/NF-κB signaling pathway due to the long term treatment by MSC-CM can suggest as one of the important reasons of reduction of inflammatory symptoms in the chronic phase of Neuroinflammation except reduction of serum TNF-α levels.

Moreover, our previous studies have shown that central and peripheral inflammatory mediators can phosphorylate and activate P38MAPK in the spinal cord; therefore, they can induce and sustain inflammatory symptoms during RA (6). Pp38MAPK contributes in the actuation of NF-κB which adjust the gene expression of wide range of inflammatory mediators (23-25). Additionally, NF-kB augments the expression of factors which are important in the adjustment of cellular proliferation, cell-cycle progression and apoptosis. NF-kB signaling pathways mediate critical events in the inflammatory responses via chondrocytes, leading to progressive extracellular matrix damage and cartilage destruction. In rat's chondrocytes, NF-kB and P38MAPK paths were considered to mediate inhibition of type II collagen and link protein gene expression via TNF-α (26). Mounting evidence has shown that NF-κB and P38MAPK are prominent regulator of the inducible expression of key pro-inflammatory cytokines, and activated NF-κB and P38MAPK have been demonstrated in great number of debilitating inflammatory disorders, namely RA (27). In the case of persistent pain states, mounting evidence has shown that various extracellular signals are capable of activating the NF-κB and P38MAPK pathways, namely TNF-α which is itself expressed by NF-κB and P38MAPK-dependent mechanisms in macrophages. Ergo, inhibition of intracellular signaling pathway molecules can decline the TNF-α level and conversely (25). Zuoning et al. have shown that NF-kB expression is eminent in the rat's synovium tissue with collagen-induced arthritis and was localized to the intimal lining as early as 3 days after arthritis induction (28). In light of the principal role of these factors in inflammatory pain, the identification of inhibitors of intracellular signaling pathway molecules should bring forth novel therapeutics for the treatment of inflammatory disease (27). In this study, we set out to determine the degree of expression of the active forms of spinal NF-kB and P38MAPK by using western blotting technique. In this regard, the results of this study also showed that phosphorylation of P38MAPK and NF-κB in the rat's spinal cord in the CFA group in different days of the study significantly increased. Studies have also shown that NF-κB and P38MAPK inhibitors are effective in reducing hyperalgesia and edema caused by inflammation (29, 30).

In conclusion, our study showed that MSC-CM administration significantly reduced the hyperalgesia and paw edema during both acute and chronic phases of CFA-induced arthritis, which was aligned with reduction of serum levels of TNF-α and spinal NF-κB and P38MAPK expression. Based on the results of this research, it is expected that anti-inflammatory effects of long term treatment by MSC-CM can be mediated via inhibition of lumbar spinal cord P38MAPK and NF-κB expression and signaling pathways. Moreover, the potent anti-edematogenic and anti-hyperalgesic effects, and principally the anti-inflammatory effects of MSC-CM, raise the possibility to be a good candidate for the control of inflammatory pain. Further clinical studies are needed to evaluate the anti-hyperalgesic and inflammatory effects of MSC-CM compared to synthetic drugs. We have to emphasize that the mechanisms that allow the CNS to modulate peripheral inflammatory responses are largely unidentified then, involved anti-inflammatory pathways during long term treatment with MSC-CM need more investigations.

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Conflict of Interests
The authors declare that they have no conflict of interest.

Authors' contributions
Study concept and design, Jalal Zaringhalam; drafting the manuscript and acquisition of data, Vida Nazemian; analysis and interpretation of data, Homa Manaheji; critical revision of the manuscript for important intellectual content, Ali Mohammad Sharifi.

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