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**Original Article**

# Chronic exercise reduces astrocytic c-Fos and CCL2 via conditioned serum and cerebrospinal fluid

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## Abstract

Inflammation, a critical immune response to infection and tissue damage, is mediated by pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), which upregulate the expression of cellular proto-oncogene Fos (c-Fos) and chemokine ligand 2 (CCL2). Chronic exercise has been shown to exert systemic anti-inflammatory effects, yet its impact on astrocytic inflammatory signaling remains unclear. The aim of this study was to investigate whether chronic exercise modulates astrocytic expression of c-Fos and CCL2 through factors present in conditioned serum and cerebrospinal fluid (CSF). Male wistar rats were assigned to an exercise group (progressively increased swimming, five times per week for four weeks) or a sedentary control group. Conditioned serum and CSF were collected and applied to astrocyte cultures with or without TNF-α induction. After 120 minutes, c-Fos and CCL2 expression were quantified using western blot analysis. Conditioned serum and CSF from exercise rats significantly reduced TNF-α induced c-Fos and CCL2 expression compared with controls. These findings suggest that chronic exercise may attenuate neuroinflammatory responses by modulating astrocytic expression of c-Fos and CCL2. The parallel reductions observed in both serum and CSF indicate that exercise-induced circulating factors may possess anti-inflammatory properties within neural environments. This study provides preliminary in vitro evidence for mechanistic link between chronic exercise and reduced neuroinflammation, underscoring the need for in vivo validation and translational research to assess therapeutic potential.

**Keywords**: Inflammation, chronic exercise, c-Fos, CCL2, cerebrospinal fluid

## Introduction

Damage to the central nervous system (CNS) triggers a complex multicellular response involving both neuronal and non-neural cells, with immune and inflammatory mechanisms playing critical roles [1,2]. Epidemiological studies highlight the global burden of neuroinflammatory disorders; for example, multiple sclerosis affects approximately 2.8 million people worldwide, while Alzheimer's disease impacts over 55 million individuals, underscoring the urgent need for effective interventions targeting neuroinflammatory pathways. Astrocytes, the most abundant glial cells in the vertebrate CNS, are key contributors to this process. Beyond their structural functions—such as forming the glial-limiting membrane that separates neural from non-neural tissue [3] and supporting blood-brain barrier integrity [4]—astrocytes regulate



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extracellular ion homeostasis, provide metabolic support to neurons, and facilitate repair following CNS injury [5]. Notably, they also play a pivotal role in modulating innate immune responses within the CNS [6], though their exact functions depend on the context and timing of activation [7].

In response to injury or disease, astrocytes produce a wide range of inflammatory mediators, including cytokines, chemokines, and prostaglandins [8]. These responses can amplify neurodegenerative processes in conditions such as ischemic stroke, traumatic brain injury, and multiple sclerosis [8]. Among key inflammatory mediators, chemokine ligand 2 (CCL2) and the transcription factor c-Fos have been implicated in CNS pathology. CCL2, expressed by astrocytes and microglia, is upregulated in neuroinflammatory conditions such as traumatic brain injury, multiple sclerosis, and HIV-associated neuroinflammation [9,10,11], where it promotes leukocyte recruitment and exacerbates neural damage [10]. Similarly, c-Fos activation in astrocytes and neurons has been linked to nociceptive signaling and glial proliferation following inflammatory stimuli [12,13]. Targeting these molecules may offer therapeutic benefits, as evidenced by studies showing improved outcomes following inhibition of CCL2 or c-Fos in animal models of neuroinflammation [14].

Exercise has emerged as a non-pharmacological strategy to mitigate neuroinflammation [15,16]. Proposed mechanisms include exercise-induced modulation of cytokine production [17], enhancement of blood-brain barrier function [18], reduction of oxidative stress, and the release of anti-inflammatory myokines such as IL-6 and IL-10 from skeletal muscle [16,17,19]. Exercise has also been shown to increase the number of astrocytes, which may contribute to neural repair and rehabilitation [20]. Epidemiological studies suggest an inverse relationship between physical activity levels and markers of systemic inflammation [21,22], although direct population-based evidence specific to CNS inflammation remains limited. Importantly, exercise may promote the release of circulating factors in biological fluids such as serum and cerebrospinal fluid that suppress neuroinflammatory pathways [23,24], but the identity and mechanisms of these factors remain poorly characterized.

Despite evidence that exercise can alter the composition of systemic and CNS biofluids, few studies have directly investigated how exercise-modified serum and CSF influence astrocyte-mediated inflammation. Furthermore, while both serum and CSF are known to carry exercise-induced factors [23,24], their relative efficacy and mechanisms of action remain unclear. This gap is significant given that CSF more accurately reflects the CNS biochemical environment, whereas serum represents systemic changes. Therefore, the aim of this study was to determine whether conditioned serum and CSF from chronically exercised rats reduce c-Fos and CCL2 expression in TNF-α-stimulated astrocytes, to compare the anti-inflammatory effects of serum and CSF to evaluate their potential differential roles, and to provide preliminary mechanistic insight into how exercise-induced factors in biofluids may modulate astrocyte reactivity.

# Methods

## Chemicals and reagents

Complete catalog numbers and research resource identifiers (RRIDs) for materials used in this experiment were as follows: Immobilon®-FL PVDF membrane pore size 0.45 purchased from Millipore Darmstadt, Germany (05317-10EA); anti-CCL2 antibodies purchased from Biorbyt, Cambridge, United Kingdom (orb36895); anti-c-Fos antibody (ABE457 ) and goat anti-rabbit IgG antibody, HRP-conjugate (12-348 ) purchased from Sigma-Aldrich, Massachusetts, United States; anti-mouse IgG HRP conjugate (ab6728) and recombinant rat TNF alpha protein (ab201903) purchased from Abcam Massachusetts, United States; DMEM/F12 medium purchased from Gibo Montana, United States (Gibco #11320033, RRID:AB_2864777); glial Mouse cell line C575 purchased from the National Cell Bank (NCBI); all other materials and reagents purchased from Merck (Darmstadt, Germany).

## Animals and ethics

Thirty male Wistar rats (200–220g) were purchased from the Experimental Studies Center of Iran University of Medical Sciences. The rats were housed at 24±2°C under a 12/12 h light/dark

cycle with ad libitum access to food and water. Animals were randomized into exercise (n=15) and sedentary (n=15) groups, and researchers were blinded to group assignments during intervention and outcome assessments. Humane endpoints included weight loss >20%, lethargy, or respiratory distress, though no animals met these criteria. For invasive procedures, rats were anesthetized with ketamine/xylazine (100/10 mg/kg i.p).

At the end of the experimental period, blood and CSF samples were collected under anesthesia and in sterile conditions by cardiac puncture and cisterna magna puncture, respectively, to obtain serum and CSF. Samples from exercised animals were considered conditioned serum/CSF, whereas samples from sedentary animals served as controls. All samples were aliquoted and stored at −80°C until analysis. Euthanasia was performed under deep anesthesia by cardiac puncture followed by cervical dislocation.

## Swimming training (ST) protocol

The exercise group underwent a pre-training adaptation process, which involved daily swimming sessions for three consecutive days in water maintained at 31±1°C. Sessions were conducted between 9 AM and 12 PM [25], and were supervised for signs of exhaustion, including impaired swimming or sinking.

The exercise protocol consisted of four weeks of swimming, with animals trained five days per week, twice daily (morning and evening), and resting for two days each week. The initial swimming duration was five minutes per session, and was increased by five minutes every two days until a maximum of 30 minutes was reached for both morning and evening sessions. This duration was then maintained constant for the remainder of the training period (**Figure 1**). Animals in the control group did not participate in any swimming exercises.

## Sample collection and quality control

All serum and CSF samples underwent rigorous quality control prior to use. Forty-eight hours following the final swimming session, under anesthesia with ketamine/xylazine (100/10 mg/kg i.p), CSF was collected from the cisterna magna using sterile 27G needles, centrifuged (2000 × g, 5 min), and screened for blood contamination. Serum was obtained via cardiac puncture, centrifuged (2000 × g, 5 min), and assessed for hemolysis by visual inspection and absorbance at 414 nm. Samples with >5% hemolysis were excluded. The CSF and serum from the sedentary group were collected using the same procedures. All samples were aliquoted and stored at −80°C to prevent repeated freeze-thaw cycles and minimize protein degradation. To maintain biological relevance and account for individual variability, serum and CSF samples from each animal were processed and analyzed individually, thereby preserving inter-individual variability and preventing pseudoreplication.

## Cell culture

C575 cells, predominantly astrocytes, were procured from the Pasteur Institute. The murine C575 astrocyte cell line was selected for its well-characterized inflammatory response and experimental practicality. Cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. For experiments, cells at passage 3 were used. The culture flasks were incubated at 37°C with 5% $pCO_2$ of 5 cm/Hg for 24 hours. Cells reaching >80% were deemed ready for passage.

The supernatant was subsequently discarded, and 2 mL of trypsin was added to each flask for two minutes. After detachment, trypsin was neutralized with 2 mL of FBS. Cells were harvested into a 15 mL Falcon tubes and centrifuged at 1200 rpm for 10 minutes. The supernatant was removed, and the pellet was resuspended in 5 mL of culture medium. Cells were then transferred into T-75 flasks, with 1 mL of cell suspension per flask, followed by 17 mL of culture medium and 2 mL of FBS. During the third passage, the culture medium was replaced, and flasks were incubated for an additional 24 hours. Cell culture treatments were applied in randomized order. Investigators were blinded to group assignments during sample processing, treatment application, and data collection. Sample codes were revealed only after completion of statistical analyses.

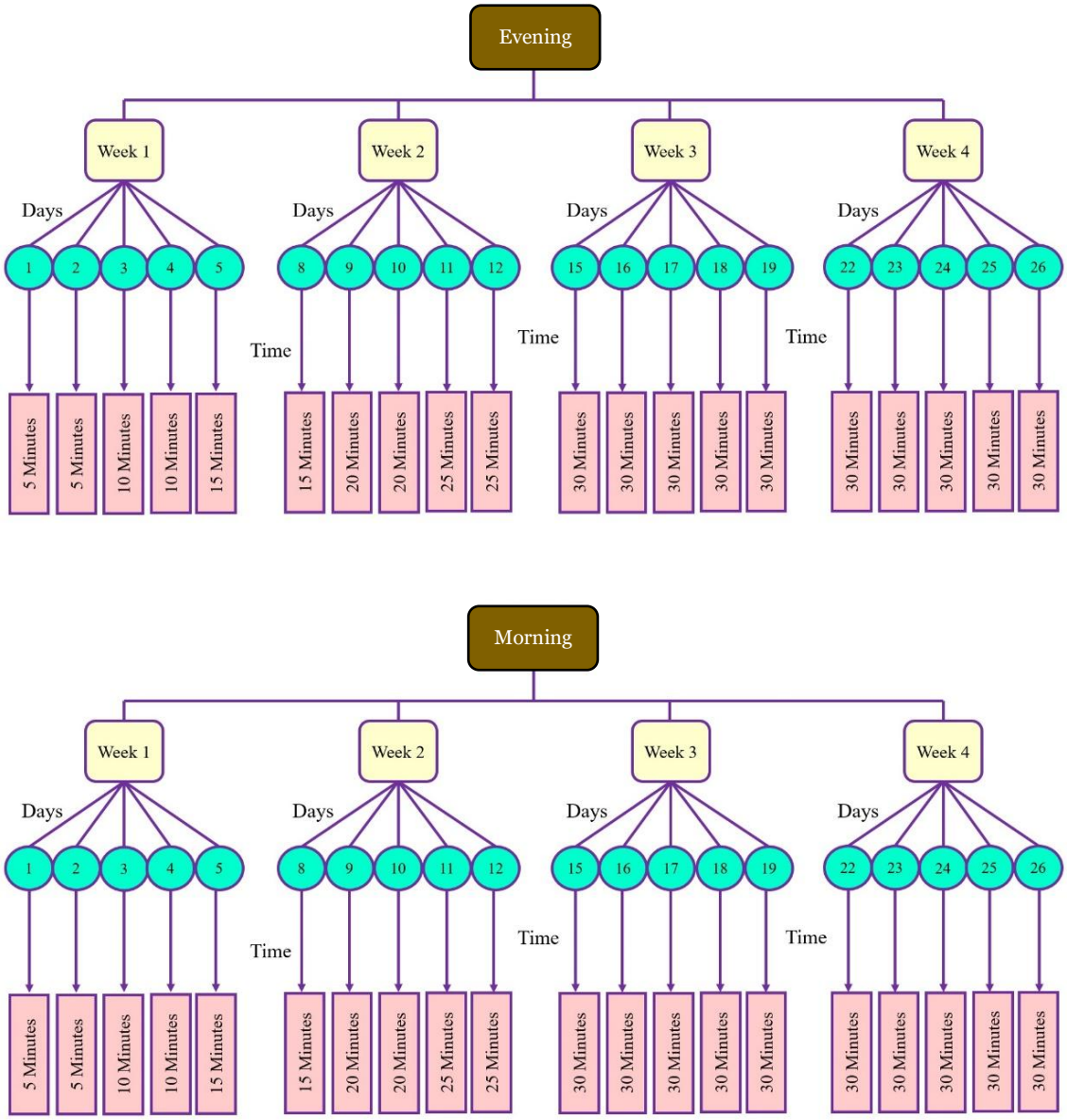


Figure 1. Four-week swimming protocol training in exercised rats.

## The experimental groups

Astrocyte cultures were divided into experimental groups based on exposure to CSF, serum, or controls. In the CSF group, cultures were treated as follows: (C1) astrocytes + 10 ng TNF-α + 1400 µL conditioned CSF of rats with physical activity; (C2) astrocytes + 10 ng TNF-α + 1400 µL CSF of rats without physical activity; (C3) astrocytes + 1400 µL conditioned CSF of rats with physical activity; and (C4) astrocytes + 1400 µL CSF of rats without physical activity. In the serum group, cultures received: (S1) astrocytes + 10 ng TNF-α + 1400 µL conditioned serum of rats with physical activity; (S2) astrocytes + 10 ng TNF-α + 1400 µL serum of rats without physical activity; (S3) astrocytes + 1400 µL conditioned serum of rats with physical activity; (S4) astrocytes + 1400 µL serum of rats without physical activity. Control groups included: (CON1) astrocyte culture flask and (CON2) astrocyte culture flask + 10 ng TNF-α.

Each experimental group contained four flasks. The exposure times were 10 minutes for TNF-α and 210 minutes for CSF or serum, with the TNF duration selected based on previous studies [26-28]. Before adding CSF or serum, the TNF-α was removed by washing with phosphate-buffered saline (PBS), after which CSF or serum from exercised or sedentary animals was added to the flask. Following 120 minutes of incubation, the media were removed, cells were washed with 2 mL of culture medium, and the flasks were vigorously shaken and rewashed. Cells were then detached using 0.25% trypsin-EDTA (5 min at 37°C). The resulting cell suspensions

were centrifuged at 2000 g for 10 minutes at 4°C. The supernatant was discarded, and 1 mL of culture medium was added to each tube. Cells were incubated at −4°C for 30 minutes, transferred to −20°C for one hour, and finally stored at −80°C for future evaluation of the proteins.

### Protein extraction

The evaluation of CCL2 and c-Fos expression in C575 cell lysate was conducted via immunoblotting. Protein extraction was performed at 4°C using an ice-cold lysis buffer, which contained 100 mM sodium orthovanadate X-200 in 20 mM Tris–HCl (pH=8), 137 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), a 1:1000 dilution of protease inhibitor cocktail, 1% NP-40 X10, 10% Glycerol, 10µg/mL Aprotinin, and 1µg/mL leupeptin. Cells were incubated in the buffer for one hour at 4°C. Following this, supernatants were collected, and protein concentrations were ascertained using Bradford assays.

### Western blotting

In the process of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), equivalent quantities (90 µg per sample) were loaded onto 10% polyacrylamide gels and separated via electrophoresis. The proteins were then electro-transferred onto polyvinylidene fluoride (PVDF) membranes, which were subsequently blocked for one hour at room temperature in 5% skim milk and 0.1% Tween-20 prepared in Tris-buffered saline. Membranes were then incubated overnight at 4°C with primary antibodies against the target proteins, followed by incubation with appropriate secondary antibodies for one hour at room temperature. β-actin served as the internal standard for the Western blot analysis. Densitometric quantification of target proteins was normalized to β-actin. For protein detection, the following antibodies were used: anti-CCL2 (1:1000) with anti-rabbit IgG peroxidase conjugate (1:1000), anti-c-Fos (1:500) with anti-mouse IgG HRP conjugate (ab6728, 1:2000); and anti-β-actin (ab8227, 1:1000) with anti-rabbit IgG peroxidase conjugate (ab6154, 1:1000). Bands were visualized using an enhanced chemiluminescence (ECL) kit according to the manufacturer's instructions, and the results were quantified using the Gel-Pro analyzer imaging software. Band intensities were quantified using automated ImageJ software with background subtraction performed using the rolling ball algorithm (radius=50 pixels), thereby minimizing subjective bias. Sample codes were revealed only after completion of statistical analysis. Three independent biological replicates were performed, each representing astrocyte cultures prepared from separate cell batches. For each biological replicate, technical duplicates (two Western blot runs per sample) were conducted to ensure reproducibility. Statistical analyses were performed based on biological replicates (n=3).

### Statistical analysis

Data were presented as mean±SEM. Satistical analyses were performed using SPSS software, version 16.0 (IBM Corp., Armonk, USA). One-way analysis of variance (ANOVA) was employed for all data sets, followed by Tukey's post hoc test. A $p$-value of less than 0.05 was considered statistically significant.

## Results

### Cell line selection and cross-species considerations

Although cross-species experimentation has inherent limitations, this approach is supported by the high functional conservation (>90% homology) of key inflammatory pathways across rodent species and by previous studies that have successfully utilized this model in neuroinflammatory research [1]. Phase-contrast micrographs further confirmed healthy C575 astrocyte morphology after four days in culture (**Figure 2**).

### The impact of conditioned CSF on CCL2 expression levels

The effect of rat CSF, obtained from exercised and sedentary animals, on TNF-α-induced alterations in CCL2 expression in astrocyte cultures is presented in **Figure 3**. CCL2 expression in astrocytes treated with TNF-α and CSF was compared with inflamed astrocytes without CSF (CON2). Similarly, expression in non-inflamed astrocytes treated with CSF was compared with non-inflamed controls (CON1). One-way ANOVA revealed a significant difference in mean CCL2 protein levels among groups (df=5; F=17.40; $p$<0.0001).

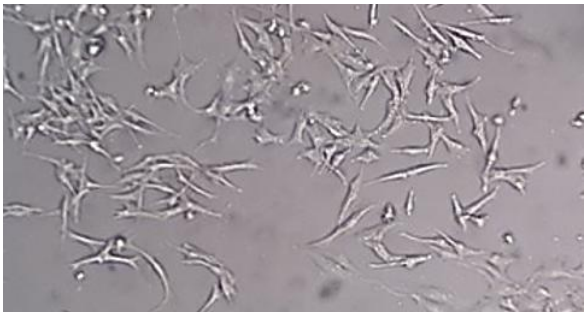

Figure 2. Phase-contrast micrographs of C575 astrocyte cells after four days in culture (200×).

Further analysis confirmed that treatment with TNF-α alone (CON2) significantly increased CCL2 levels (131.43±2.12, $p$=0.0001) compared with CON1 (101.3±3.22). This finding highlights the pro-inflammatory effect of TNF-α on astrocytes (**Figure 3A**).

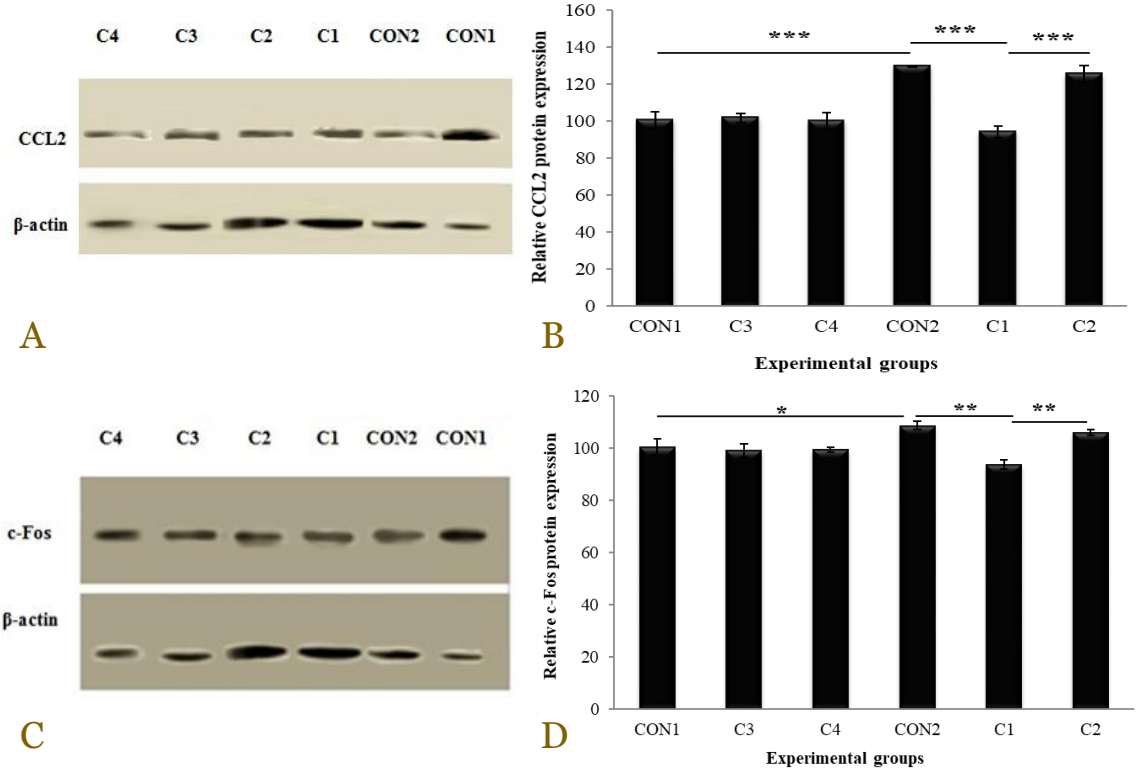


Figure 3. Effects of CSF from rats with or without chronic exercise training on TNF-α-induced CCL2 and c-Fos expression in astrocyte culture media, assessed by western blot. β-actin level was used to normalize the density of each band. (A) Western blot bands of CCL2 and β-actin; (B) relative CCL2 protein expression; (C) western blot bands of c-Fos and β- proteins; (D) relative c-Fos protein expression. Data are presented as mean±SEM. $^{***}p$<0.001, $^{**}p$<0.01, $^{*}p$<0.05 were used to express statistical significance among groups.

Western blot analysis showed that CCL2 expression in inflamed astrocyte cultures treated with conditioned CSF from exercised rats (C1) was significantly lower compared with CON2 (93.86±3.23 vs 131.43±2.12, $p$<0.0001). In contrast, inflamed cultures treated with CSF from sedentary rats (C2) showed no significant difference from CON2 ($p$=0.12). Moreover, CCL2 levels in the C1 group were significantly lower compared to the C2 group (125.54±4.57, $p$<0.0001). The addition of CSF, regardless of exercise status, to non-inflamed astrocyte cultures did not affect CCL2 expression, with no significant differences observed among groups ($p$=0.33; **Figure 3A** and **3B**).

## The impact of conditioned CSF on c-Fos expression levels

The effects of CSF from exercised and sedentary rats on TNF-α-induced c-Fos expression in astrocyte cultures are presented in **Figure 3**. One-way ANOVA revealed a significant difference

in mean c-Fos protein levels among groups (df=5; F=7.08; $p$=0.001). Conditioned CSF from exercised rats significantly decreased c-Fos expression in inflamed astrocytes (C1, 93.65±1.83) compared with CON2 (108.68±1.3, $p$=0.003). No significant difference was observed between the sedentary CSF group (C2) and CON2 ($p$=0.25). Notably, c-Fos expression in the C1 group was significantly lower than in the C2 group (105.97±1.13, $p$=0.005). The addition of CSF from exercised or sedentary rats to non-inflamed astrocyte cultures did not affect c-Fos expression, and no significant differences were observed compared to CON1 ($p$=0.41; **Figure 3C** and **3D**).

## The impact of conditioned serum on CCL2 expression levels

The effects of rat serum, obtained from animals with or without chronic exercise training, on TNF-α-induced alterations in CCL2 levels in astrocyte culture media are presented in **Figure 4**. One-way ANOVA revealed a significant difference in mean CCL2 protein levels among the groups (df=5; F=9.64; $p$<0.0001).

Western blot analysis demonstrated that CCL2 expression in inflamed astrocytes treated with conditioned serum from chronically exercised rats (S1) was significantly reduced compared with the CON2 group (92.79±5.17 vs 131.43±2.12, $p$<0.0001). In contrast, CCL2 expression in inflamed astrocyte cultures treated with serum from sedentary rats (S2) did not differ significantly from the CON2 group ($p$=0.16). Moreover, CCL2 levels in the S1 group were significantly lower compared with the S2 group (125.54±4.57, $p$=0.002).

The addition of serum, regardless of exercise status, to non-inflamed astrocyte cultures (without TNF-α stimulation) did not alter CCL2 expression, with no significant differences observed between groups ($p$=0.37; **Figure 4A** and **4B**).

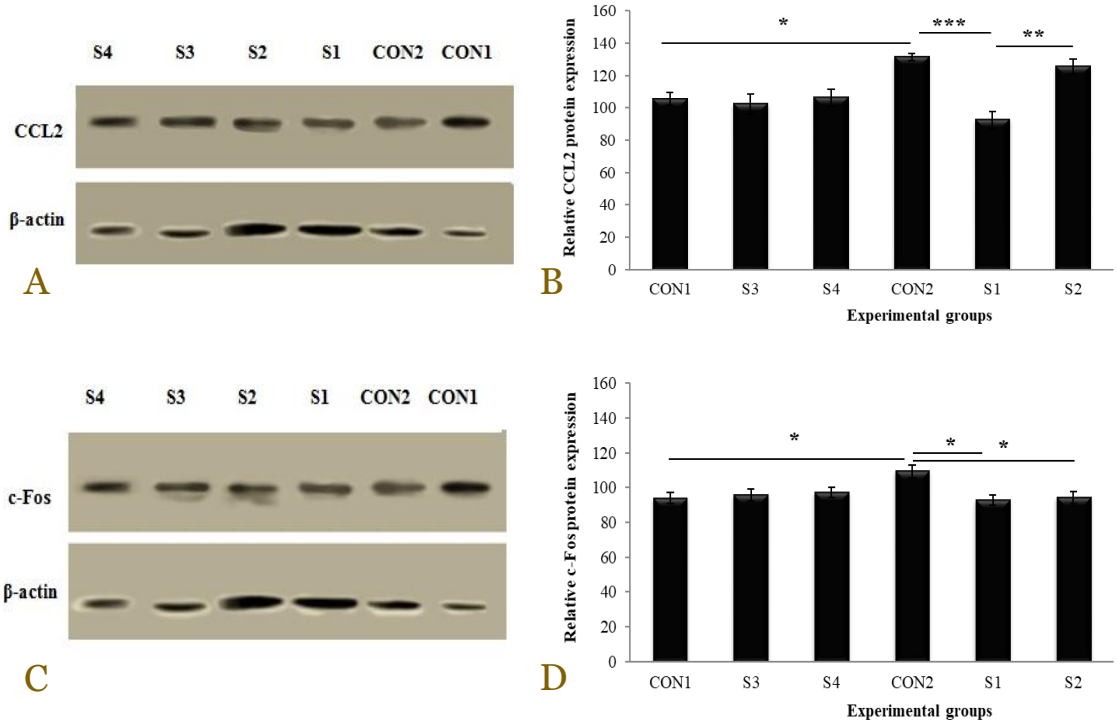


Figure 4. Effects of serum from rats with or without chronic exercise training on TNF-α-induced CCL2 and c-Fos in astrocytes culture media, assessed by western blot. β-actin level was used to normalize the density of each band. (A) western blot bands of CCL2 and β-actin; (B) relative CCL2 protein expression; (C) western blot bands of c-Fos and β-actin; (D) relative c-Fos protein expression. Data are expressed as mean±SEM. $^{***}p$<0.001, $^{**}p$<0.01, $^{*}p$<0.05 indicate statistical significance among groups.

## The impact of conditioned serum on c-Fos expression levels

The effects of serum from exercised and sedentary rats on TNF-α-induced c-Fos expression in astrocyte culture media are presented in **Figure 4**. One-way ANOVA indicated a significant difference in mean c-Fos protein levels among the groups (df=5; F=3.57; $p$=0.02). Western blot analysis revealed that c-Fos expression in inflamed astrocyte cultures treated with conditioned serum from exercised rats (S1, 92.9±2.87) was significantly lower compared with the CON2 group

(109.37±3.34, $p$=0.021). Similarly, inflamed astrocytes treated with serum from sedentary rats (S2) also showed a significant reduction compared with the CON2 group ($p$=0.038). However, no significant difference was observed between the S1 and S2 groups (94.47±4.28 vs 92.9±2.87, $p$=0.54). Furthermore, the addition of serum, regardless of exercise status, to astrocyte cultures without TNF-α stimulation did not affect c-Fos expression, and no significant differences were detected among groups ($p$=0.67; **Figure 4C** and **4D**).

### Comparison of conditioned serum and CSF on c-Fos and CCL2 expression

The impact of conditioned serum and CSF on the expression of c-Fos and CCL2 was evaluated to assess the potential anti-inflammatory effects of biofluids derived from exercised animals (**Figure 5**). The results demonstrated no significant difference between conditioned serum and CSF from chronically exercised animals in modulating c-Fos and CCL2 expression. Moreover, expressions of both markers remained unchanged in astrocyte cultures regardless of TNF-α stimulation.

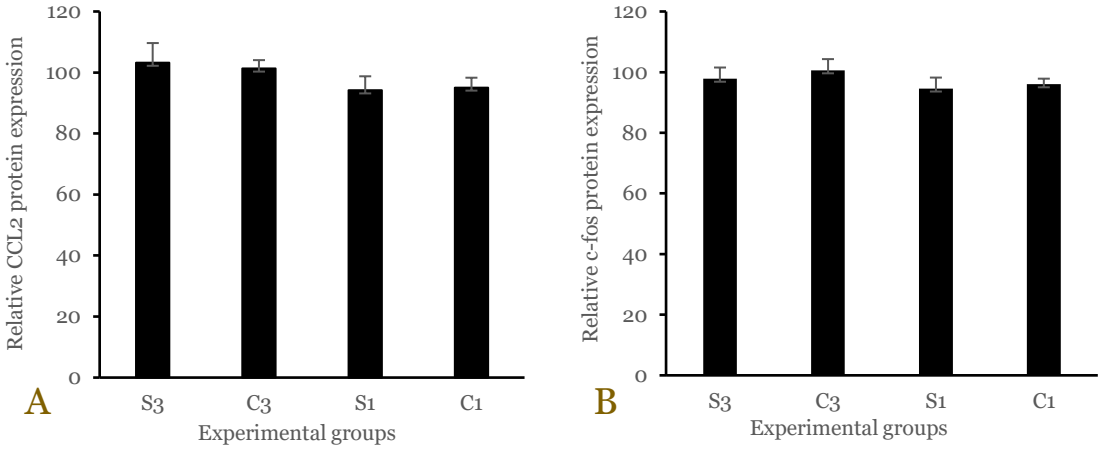


Figure 5. Comparison of the effects of conditioned serum and CSF from rats with chronic exercise training on CCL2 (A) and c-Fos (B) expression in C575 astrocyte culture media.

## Discussion

In this study, TNF-α was added to astrocyte culture media to model inflammatory processes in the CNS. The effects of conditioned CSF and serum from rats subjected to chronic exercise on the expression of c-Fos and CCL2, two key pro-inflammatory markers, were then evaluated. Statistical analysis confirmed that TNF-α significantly increased c-Fos and CCL2 expression, validating the inflammatory model. Elevated levels of these chemokines are known to disrupt astrocyte–astrocyte interactions and eventually lead to cell death [29].

The present study demonstrated that conditioned serum and CSF from exercised rats significantly attenuated TNF-α–induced increases in c-Fos and CCL2 expression. Notably, no significant differences were observed between serum and CSF, suggesting that chronic exercise produces comparable protective adaptations in both biofluids. These findings highlight the ability of exercise-induced systemic and central changes to limit the spread of inflammation.

Previous studies have shown that TNF-α plays a central role in the inflammatory cascade by promoting the release of other mediators, such as interleukin-1 (IL-1), which subsequently induces CCL2 expression [30]. Elevated CCL2 levels are common in inflamed tissues and are associated with delayed therapeutic responses. Increased c-Fos and CCL2 expression has been documented in several CNS disorders, including Parkinson's disease [31], Alzheimer's disease [32], and multiple sclerosis [33]. Anti-inflammatory strategies have been shown to reduce CCL2 and its receptor expression, and growing evidence suggests that CCL2 inhibition could be an effective therapeutic approach in neurodegenerative diseases [34]. Astrocytes play a pivotal role in cytokine regulation in the CNS and contribute significantly to neuroprotection during inflammatory states [35].

Exercise has recently gained attention as a low-cost, low-risk intervention with strong anti-inflammatory and neuroprotective properties. Progressive aerobic exercise, such as swimming, has been shown to improve depressive symptoms through its anti-inflammatory and anti-stress

effects [36,37]. Conversely, increased levels of CCL2 and other inflammatory chemokines can compromise astrocytic integrity and impair the immune system's ability to regulate inflammation [38]. Beyond the CNS, CCL2 has also been implicated in breast tumor metastasis [39] and CNS infections [40].

Consistent with prior findings, TNF-α in our study markedly elevated CCL2 in astrocytes, while exercise-conditioned fluids mitigated this effect. Nevertheless, the impact of exercise depends critically on intensity, duration, and type: moderate-intensity activity strengthens immune function by upregulating anti-inflammatory cytokines, whereas vigorous or abrupt increases in intensity can promote inflammation [41-44]. Indeed, sudden changes in training load have been shown to exacerbate muscle inflammation [45,46].

Chronic moderate-intensity exercise exerts neuroprotective effects via multiple mechanisms, including suppression of TNF-α signaling, reduction of oxidative stress, and enhanced neuronal survival and regeneration [47-49]. In Parkinson's models, progressive aerobic training has improved motor function, likely by enhancing synaptic connectivity at neuromuscular junctions [50]. These findings emphasize that exercise intensity and duration are critical modulators of its neuroprotective effects.

The expression of c-Fos, a marker of neuronal activation, is also regulated by exercise parameters. Morikawa reported intensity-dependent increases in hypothalamic c-Fos expression regardless of duration [51]. Lee demonstrated that treadmill exercise induced transient elevations in c-Fos, peaking on day seven and then declining [52]. Similarly, Tsai and colleagues showed that both acute and long-term mild-intensity treadmill running increased c-Fos expression in the limbic system, with longer interventions producing more enduring effects [53]. Forced swimming has also been shown to elevate c-Fos expression, an effect that can be blocked by glucocorticoid receptor antagonists [54]. Beyond molecular markers, physical activity improves neuropsychiatric outcomes, promotes neurogenesis, enhances mitochondrial function, and reduces neuronal apoptosis [55].

The reduction of c-Fos and CCL2 expression observed in this study following exposure to exercise-conditioned fluids is consistent with the anti-inflammatory effects reported in previous studies [30,37]. However, our experimental design did not allow us to identify the precise factors responsible for this effect. Potential candidates include myokines (e.g., IL-6), neurotrophins (e.g., BDNF), metabolites (e.g., lactate), or other exercise-induced molecules [42,43]. Future work should quantify these components in serum and CSF and examine their individual and synergistic roles in modulating astrocyte inflammation. In addition, while the present study focused on two inflammatory markers (CCL2 and c-Fos), this narrow scope does not capture the broader spectrum of exercise-induced immunomodulation. Expanding analyses to additional cytokines (e.g., IL-6, TNF-α, IL-1β), chemokines, and signaling pathways will provide a more comprehensive picture.

The 120-minute exposure time was selected based on experiments demonstrating that peak inflammatory marker expression (CCL2 and c-Fos) occurs at this time point following TNF-α stimulation in astrocyte culture system [56,57]. While this acute exposure captures the immediate modulatory capacity of exercise-conditioned fluids, we acknowledge that it may not fully reflect the chronic effects of exercise-induced factors. Future studies employing longer-term exposure models will be valuable to investigate the sustained effects of exercise-conditioned biofluids. Both conditioned serum and CSF from exercised animals reduced c-Fos and CCL2 expression in inflamed astrocytes, whereas samples from sedentary animals had no effect. These findings suggest that chronic, progressively intensified exercise induces systemic and CNS adaptations—including skeletal muscle, metabolic, and cardiorespiratory systems [58]. Unlike strength-based exercise, which is often associated with increases in inflammatory factors, chronic aerobic exercise with gradual progression appears to suppress inflammation and stimulate anti-inflammatory pathways [59]. Given the intact blood-brain barrier in healthy animals, it is unlikely that changes in one fluid directly mirror those in the other. Instead, exercise likely exerted parallel effects on serum and CSF, leading to similar modulation of astrocytic inflammation.

Several limitations should be considered. First, this in vitro model cannot fully replicate the complexity of neuroinflammation in vivo. Second, the short exposure time (120 minutes) does not capture chronic inflammatory dynamics relevant to neurodegenerative disease. Third,

although a murine astrocyte line was used with rat-derived fluids, cross-species applicability is supported by prior studies showing functional conservation of key inflammatory mediators (e.g., TNF-α, IL-6) and similar astrocytic responses to TNF-α challenge across rodents. Nevertheless, future work should validate findings in rat-specific models. Finally, although the swimming protocol was standardized, biochemical indicators of exercise intensity (e.g., blood lactate) and stress response (e.g., corticosterone) were not directly measured. Incorporating these parameters in future studies will provide a more comprehensive characterization of the exercise stimulus.

Despite these limitations, this study contributes to the growing body of evidence supporting exercise-induced immunomodulation. The comparable effects of serum and CSF suggest that similar anti-inflammatory mechanisms may operate in both peripheral and central compartments. Future research should determine whether these in vitro findings translate to in vivo models of neuroinflammation and whether comparable effects are observed in clinically relevant settings.

## Conclusion

In vitro, serum and CSF from chronically exercised rats can reduce the expression of pro-inflammatory markers c-Fos and CCL2 in TNF-α-stimulated astrocytes. These findings suggest that exercise-induced modifications in biological fluids may contribute to the anti-inflammatory effects observed in vivo, although the specific mediating factors remain unidentified. Further research is needed to characterize the bioactive components responsible for these effects, determine their relevance in animal models of neuroinflammation, and evaluate their potential translational applications for human neuroinflammatory disorders.

### Ethics approval

Experimental research protocol on animals was approved by the ethics committee of Iran University of Medical Sciences (IR.IUMS.REC.1399.1383) and complied with ARRIVE guidelines. All procedures were strictly conducted in accordance with the code of ethics.

### Acknowledgments

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### Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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### Underlying data

Data are available on reasonable request.

### Declaration of artificial intelligence use

This study used artificial intelligence (AI) tools such as ChatGPT in the language refinement to improve grammar, sentence structure, and readability of the manuscript. We confirm that all AI-assisted processes were critically reviewed by the authors to ensure the integrity and reliability of the results.

## How to cite

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