

Research Paper:

Effect of Nano-eugenol and Aerobic Exercise Against the Streptozotocin Toxicity and Inflammatory Mediators P38-MAPK, NPY, and A-R α 2A in the Dorsal Root Ganglia of Diabetic Rats



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ABSTRACT

Background: The aim of this study was to investigate the effects of nano-eugenol combined with aerobic exercise against the streptozotocin toxicity and inflammatory mediators P38-MAPK, NPY and A-R α 2A in the dorsal root ganglia of diabetic rats.

Methods: Twenty-five, 8-week-old Wistar male rats were divided into 5 groups: 1) normal control group (normal model); 2) diabetic control group (diabetic model); 3), diabetic + exercise group (diabetic+exercise model); 4) diabetic group + nano-eugenol (diabetic+nano model); and 5) diabetic + exercise + nano-eugenol (diabetic+exercise+nano model). Diabetes was induced in the experimental groups 2 through 5 by the intraperitoneal injection of streptozotocin at 4mg/100 grams of the rats' body weight. The nano-eugenol supplement was also gavaged into the supplement groups 4 and 5 only. Groups 3 and 5 exercised progressively at a speed of 8 to 20 meter/min for 5 to 30 min, five days a week over the 8-week study duration.

Results: The diabetic rats that exercised and were treated with the nano-eugenol, showed a significant decrease in P38-MAPK gene expression compared to the normal model group ($P=0.001$). The study of the therapeutic modalities also showed that only the diabetic + exercise + nano-eugenol group showed a significant increase in NPY and A-R α 2A genes compared to the normal model ($P=0.001$).

Conclusion: Based on the results, the use of nano-eugenol supplementation combined with aerobic exercise is likely to be effective in controlling the neurological damages due to diabetes by negatively regulating the P38-MAPK gene while positively regulating the NPY and A-R α 2A genes in the DRG region.

Keywords: Aerobic exercise, Adrenoreceptor α 2A, Diabetic neuropathies, Neuropeptide Y, P38 Mitogen-Activated Protein Kinases

Introduction

Despite much therapeutic advancements made in the medical world, type-II diabetes is still on the rise among populations, especially in the developing countries [1].

It is estimated that the number of people with type-II diabetes will approach 592 million by 2035, up from 382 million in 2013 [2]. Lack of physical activity, inappropriate diet, and increased life stressors weaken the body's immune system and can lead to diabetes, which has affected the lives of a large population worldwide [1, 2].

The pathological conditions arising from diabetes can involve almost all human organs, including renal, cardiac, retinal, blood and neurological systems. Of note, peripheral neuropathy is one of the major neurological conditions secondary to diabetes that emerges years following the initial pathology [3]. Diabetic neuropathy and its various clinical features have been the subjects of numerous studies for years; however, the pathogenesis is not completely clear to date [4]. It is established that the high blood glucose in diabetes is the initial cause of most if not all of the associated pathologies. These include increased oxidative stress, local inflammation, polyol pathway activation, heightened protein kinase-C synthesis and glycosylation biproducts, hypoxia and ischemia in the Central Nervous System (CNS), and reduced linoleic acid and growth factors [5]. Clearly, these events promote oxidative stress and inflammatory response; however, structural damage to the Dorsal Root Ganglion (DRG) is believed to a major neurological health concern [6].

Researchers have examined a number of inflammatory factors prevalent in diabetes and their contributions to the pathogenesis of certain human neurological conditions [6]. Among these factors, the enzyme p38-MAPK is believed to be associated with neuropathic pain and damages to nervous structures in diabetes, although their precise role in the DRG is not well known [6, 7]. This enzyme is activated via phosphorylation in response to oxidative stressors and is a candidate toward drug development for the treatment of peripheral inflammatory conditions, such as diabetic neuropathy [7]. There is evidence to suggest that p38-MAPK is generated during inflammatory conditions secondary to oxidative stress, cell apoptosis and metabolic disorders. It has been further suggested that the inhibition of this enzyme can lead to sufficient anti-inflammatory effects to relieve the neuropathic pain that many patients with diabetes suffer from [7, 8]. This enzyme is involved in the intracellular signaling transduction, in which Neuropeptide-Y (NPY) serves as a proinflammatory factor associated with both the neuropathic pain and peripheral nerve injury in diabetes [9]. Although the expression of NPY is increased in DRG and the spinal cord in neuropathic pain, the rise in the two regions reflects distinct mechanisms. The increased expression of NPY in DRG may contribute to neuropathic pain, since injection of NPY into the DRG areas increases sensory hypersensitivity and this response is abolished by Y2 receptor antagonist [10].

Recent studies have identified novel molecules in the DRG region after nerve injury that are also implicated in generation and maintenance of pain [11]. They suggest that both down-regulation of opioid receptors and up-reg-

ulation of cholecystokinin B receptors may contribute to the attenuated analgesic effects of opioids in neuropathic pain. The up-regulation of Adrenoceptor-2A (AR-2A) and Neuropeptide Y (NPY) Y2-R enhances pain perception through sympathetic inputs [12]. It is believed that the damaging effects of these inflammatory factors in addition to Reactive Oxygen Species (ROS) on the nervous system is clinically important, since the regenerative capacity and blood supply in this region is limited [13]. A potential approach to prevent the nervous tissue damages caused by inflammatory factors and ROS in diabetes is the use of natural supplements containing phenolic acid, such as eugenol, also called clove oil [14]. This fragrant food additive has shown potent antioxidant, antitoxic and antibacterial effects, and is believed to protect nervous tissue against pathologic damages and cell apoptosis in diabetic cases [15]. Epidemiologic studies indicate that natural phenolic compounds, found in many foods and fruits, offer therapeutic or preventive effect against diabetes [15]. This supplement, especially in a nano-formulation, can enhance its penetration into cells and be used as an antidiabetic drug with lasting effect [16].

Further, regular physical activity is known to enhance the immune system's ability to protect against inflammatory conditions, particularly in the nervous system [15, 16]. In this context, aerobic exercise has been shown to relieve neuropathic pain in diabetics, likely through the inhibition of NPY and

A-R α 2A and/or increased synthesis of neurotrophic factors in nervous tissue [16]. Natural supplements or physical activities have been studied separately in the prevention of nervous tissue damages due to diabetes [17, 18]. The use of nano-eugenol as emulsions combined with regular physical exercising may be an effective therapeutic approach in the management of neuropathic pain in diabetic patients, which has not been studied to date. The current study has investigated the effect of aerobic exercising combined with the administration of a nano-formulation of eugenol on the destructive changes in the DRG and levels of P38-MAPK, NPY and A-R α 2A genes in diabetic rats.

Materials and Methods

Study design: Twenty-five healthy adult male Wistar rats, 8-week- old, weighing 200-250g were purchased from the Pasteur Institute, Tehran, Iran. They were acclimatized to the laboratory environment under controlled conditions of temperature (22 \pm 3°C), humidity (45%) and lighting (12 hr. light-dark cycle; 6 a.m. to 6 p.m.) over one week prior to the experiments. They were kept

in plexiglass cages (43 length x 27 deep x 25 cm height) with a screen gate.

Ethical guidelines: Rats were free to move around the cages with free access to food and water ad libitum. This study fully observed the institutional guidelines on ethics and the principles of Helsinki declaration with respect to the use and treatment of experimental animals. All experimental steps were designed to ensure minimal pain and suffering to the rats. The study protocol was approved by the Research Ethics Committee, Islamic Azad University, Khorasgan branch, Isfahan, Iran (Ethics Code #: IR.IAU.KHUISF.REC.1399.046).

Animal grouping: The animals were randomly divided to five groups of 3-5 rats per group, and administered the following treatment:

- Group 1: Healthy controls (no exercise or drugs given)
- Group 2: Diabetic rats (no exercise or nano-eugenol given)
- Group 3: Diabetic rats + 5 exercise sessions/week x 8 weeks
- Group 4: Diabetic rats + nano-eugenol (2 mL) x 5 days/week x 8 weeks
- Group 5: Diabetic rats + nano-eugenol (2 mL) + exercise x 5 sessions/week x 8 weeks

Induction of diabetes: The rats in groups 2-5 were made diabetic seven days in advance of the exercise and nano-eugenol experiments by injecting them intraperitoneally a single dose of Streptozotocin (STZ) at 4mg/100 gram of the rats' body weight. The rats' blood glucose levels were measured several times one week after the streptozotocin injection and those with a blood glucose level of ≥ 250 mg/dL were designated as being diabetic and were included in groups 2-5 of the experiments [17]. Rats in group 1 were designated as healthy controls and did not receive any treatment.

Nano-eugenol preparation & treatment: Pure eugenol (4-allyl-2-methoxyethane) oil was purchased from Sigma-Aldrich (Munich, Germany) and dissolved in Dimethyl Sulfoxide (DMSO). The mixture was added slowly to Tween 20, a deionized polysorbate surfactant, and stirred to complete homogeneity. A 10% nano-particle aqueous solution was prepared gently, warmed up to 37°C and added dropwise to the eugenol-Tween mix-

ture, which was then exposed to ultrasound for 10 minutes. Two mL of the nano-eugenol solution was given to each of the rats in groups 4 and 5 by gavage [18] over five consecutive days per week at the same time during the 8-week experiment.

Exercise protocol: Both the healthy control and diabetic rats (groups 1 & 2) were kept in their cages in the laboratory without being given exercise or nano-eugenol over the study period. Rats in groups 3, 4 and 5 were given daily exercises in a running wheel at increasing weekly speed (8-20 m/min) and duration (5-30 min) over the 8-week period (Table 1). In cases when the rats slowed down or stopped running in the wheel, we tapped on the wheel or made another noise to alert them to run, but we never used electric shocks for this purpose [16].

RNA extraction: Before the genetic analysis, RNA isolation from DRG tissue samples was performed, using one-step real time Polymerase Chain Reaction (PCR) assay (QIAGEN®, Germany). For this purpose, 200µL Q-Solution (QIAGEN®, Germany) was added to each of the DRG tissue samples and stored at -80°C for 24 hours. The semi-thawed samples in the cryotubes were crushed to which 100µL chloroform was added. The tubes sat at room temperature for one minute and were centrifuged at 12000 rpm (17709 G) for 10 minutes. The supernatants, containing RNA, were gently collected and placed in microtubes, to which 1mL isopropanol was added. They were vortexed and centrifuged at 12000 rpm for 10 minutes. The supernatants were discarded and 1mL ethanol (70%) was added to the pellet in each microtube and vortexed. The tubes were then centrifuged at 7500 rpm (6026 G) for 10 minutes. The supernatants were discarded and the pellets lyophilized. These were mixed with 20µL distilled water at 60°C and the tubes were kept on a warm plate at 60°C for five minutes.

RNA levels for P38-MAPK, NPY and A-Rα2A: Upon RNA extraction from all samples at high purity, the cDNA synthesis for each sample was performed based on the supplier's protocol (Fermentas, USA). The cDNA samples were then used for the process of reverse transcription. To quantify the RNA levels for P38-MAPK, NPY and A-Rα2A, we used real time PCR method with Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as the control gene. The RNA levels for the genes were calculated based on $2^{-\Delta\Delta CT}$ formula. The primers were designed by Macrogen Company (Rockville, MD, USA), based on the genetic information from the NCBI GenBank database (NIH, Bethesda, MD, USA). The oligonucleotide sequences for the P38-MAPK, NPY, A-Rα2A and GAPDH genes are presented in Table 2.

Table 1. Rats' schedule for exercise on running wheel

Week	Running Speed (m/min)	Exercise Duration (min)
1	8	5
2	10	10
3	10	14
4	14	18
5	14	22
6	17	26
7	20	30
8	20	30

Statistical analyses: All data were presented as the Means±Standard Error of the Mean (SEM). Upon confirming the data normality on Shapiro-Wilks' test, they were statistically examined by one-way Analysis of Variance (ANOVA) with Tukey's post-hoc test on SPSS software, version 22. The minimum level of statistical significance was set at $P \leq 0.05$.

Results

Changes in mRNA Expressions

P38-MAPK gene: Changes in the expression of mRNA genes for the inflammatory factors, P38-MAPK, NPY and A-Ra2A are shown in Figures 1, 2, and 3. Expression of the mRNA for P38-MAPK in group 2 showed a significant increase compared to that of the healthy controls (Group 1; $P=0.001$). There were insignificant increases in the expression of the mRNA for P38-MAPK gene in groups where rats had exercise training (Group 3), or given nano-eugenol (Group 4) compared to that found in Group 1 (controls).

However, there was a significant decrease in the expression of mRNA for P38-MAPK in Group 5, where the rats had the three treatments combined, compared to that found in the diabetic rats (Group 2; $P=0.001$) (Figure 1).

NPY gene: Upon the analysis of the mRNA for NPY, it was noted that the induced diabetes caused a significant decrease in the DRG cells ($P=0.001$) compared to that documented for the healthy controls (Group 1). However, there were insignificant increases in the expression of mRNA for NPY gene in the DRG cells of the rats who had exercise training (Group 3), or given nano-eugenol (Group 4), or had both treatments combined (Group 5) compared to that noted for Group 2 (diabetes alone) (Figure 2).

A-Ra2A gene: Upon the analysis of the mRNA for A-Ra2A, it was noted that the induced diabetes caused a significant decrease in the DRG cells ($P=0.001$) compared to that found in Group 1 (controls).

Table 2. The primers' sequences

Gene Symbol	Primer Sequence (5' to 3')
NPY	Forward: GTACCTCAAGCCGGACATAT
NPY	Reverse: CAAGCCTTGTCTGGGGAAT
A-Ra2A	Forward: TGGTAGTTTGGTAAGGTGTGG
A-Ra2A	Reverse: TGAGTGGTGGGAAGGAGATGA
P38-MAPK	Forward: GCT TAC CGA TGA CCA CGA TC
P38-MAPK	Reverse: TTC ATT CAC AGC GAG GTT GC
GAPDH	Forward: AAG TTC AAC GGC ACA GTC AAG G
GAPDH	Reverse: CAT ACT CAG CAC CAG CAT CAC C

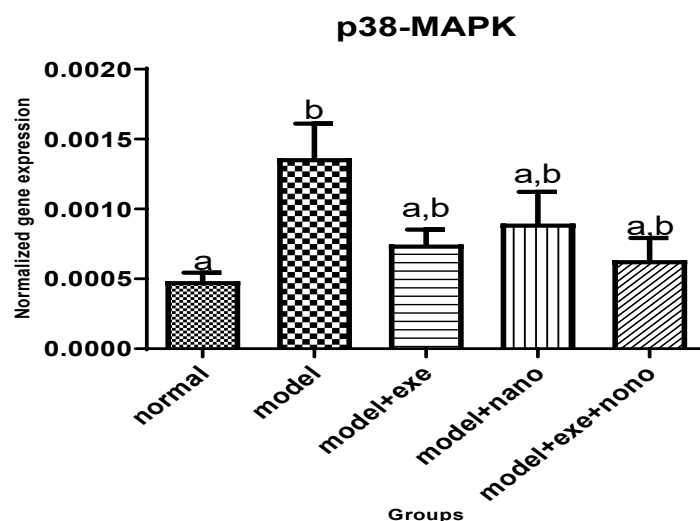


Figure 1. Changes in the expression of mRNA gene for the inflammatory factor, P38-MAPK across the study groups

pared to that of the healthy controls (Group 1). However, there were insignificant increases in the expression of mRNA for A-Rα2A gene in the DRG cells of the rats who had exercise training (Group 3), or given nano-eugenol (Group 4), or had both treatments combined (Group 5) compared to that of Group 2 (diabetes alone) (Figure 3).

Discussion

Physical activity offers a wide range of health benefits that are protective against disease processes. However, the complex mechanisms that are triggered by physical activity and lead to the reconstruction of damaged cellular

molecules are not fully understood. Improvement in degenerative nervous disorders including neuropathic pain due to diabetes is associated with regular physical activity; however, a definitive treatment or management is not yet established. The findings of the current study suggest that aerobic exercising combined with nano-eugenol supplementation is significantly more effective in controlling damages to the DRG neurons by excess inflammatory factors generated in diabetes than either approach alone.

Effect on P38-MAPK level: This study demonstrated that the P38-MAPK level had increased in the DRG area of experimental rats after the induction of diabetes under

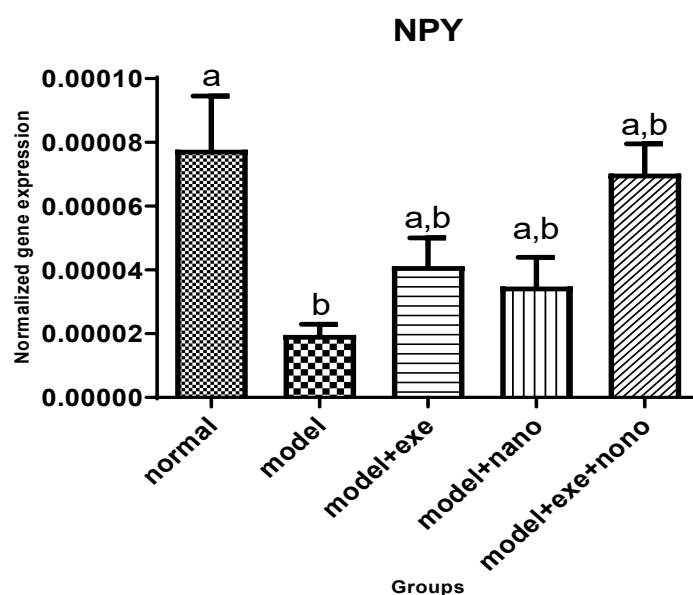


Figure 2. Changes in the expression of mRNA gene for the inflammatory factor, NPY across the study groups

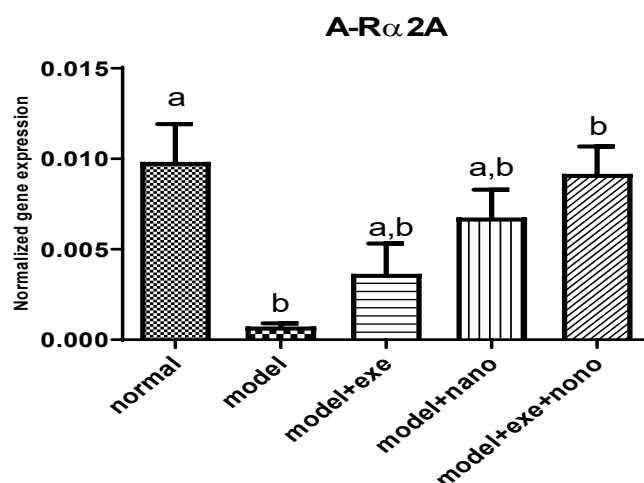


Figure 3. Changes in the expression of mRNA gene for the inflammatory factor A-Rα2A across the study groups

the toxic effect of Streptozotocin (STZ). This finding is in agreement with those suggested by previous studies conducted in animal models and humans with diabetes [19]. Given the clinical symptoms, it is likely that the increased blood glucose and ROS factors promote the activation of P38-MAPK and damage to sensory neurons in DRG region that is manifested as neuropathic pain in both experimental animals and diabetic patients. We further demonstrated that either aerobic exercise or nano-eugenol alone was able to decrease the P38-MAPK level. Interestingly, the combination of aerobic exercise and nano-eugenol proved to be even more effective than either treatment alone in the experimental rats with induced diabetes. An earlier study [20] demonstrated that the inhibition of P38-MAPK led to slowing down or prevention of the neuropathic pain and the disrupted neural conduction developed in rats after they were made diabetic by injecting them with a dose of STZ. This finding also supports the notion that the activation of P38-MAPK is likely to be the first step that triggers the neuropathic dysfunction in laboratory animals [20]. Another interesting discovery is that aerobic exercising in experimental models has led to the relief of diabetic inflammatory symptoms, likely through the inhibition of P38-MAPK in the DRG neurons [21].

Effect on NPY level: The current study findings further demonstrated that the induced diabetes significantly reduced the NPY level in the DRG neurons compared to that of the control rats. However, aerobic exercise combined with nano-eugenol supplementation significantly increased the NPY level in the DRG area of the diabetic rats, compared to those of the diabetic group that did not receive the combined treatment. The decline in NPY level was also evident in the spinal nerve roots of the

rats in the treated area of DRG, which was associated with some degrees of spinal cord dystrophy. An earlier study has demonstrated increased volume and number of sprouting nerves subsequent to moderate intensity exercise in experimental rats [22]. Also, other studies have demonstrated that exercise has led to the adaptation, increased motor units and innervation of the muscles involved in exercising [23]. It appears that aerobic exercise promotes the synthesis of NPY in the involved spinal segment thereby preventing the axonal atrophy secondary to diabetes. This effect is further enhanced if the rats had been given nano-eugenol concurrently. Although there is no experimental data available on the effect of nano-eugenol combined with concurrent exercise in experimental animals, our preliminary findings suggest that nano-eugenol provided and enhanced the level of antioxidant support such that neuronal damages due to diabetes could be reduced, repaired or prevented in experimental animals. In this context, further research is warranted to establish the cellular and molecular mechanisms by which the combination of aerobic exercise and nano-eugenol supplementation inhibit the destructive effects of diabetes in animal and human models [22, 23].

Effect on A-Rα2A level: The results from the current study demonstrated that the level of A-Rα2A in the DRG area declined significantly in the diabetic rats compared to those in the control group. However, in the rats that had received either nano-eugenol or exercise treatment, there was a significant rise in the A-Rα2A level in their DRG area. Consistently with the previous effects, the rise in the A-Rα2A level was the highest in the group that was exposed to combined exercise and nano-eugenol treatment. The decline in the A-Rα2A level in the diabetic rats is attributable to the destructive effect of diabetes on

the DRG neurons. It has been shown that $\alpha 2$ adrenergic receptors are present extensively throughout the central and peripheral nervous systems, including the DRG areas. These can be activated by various factors to generate different responses or clinical outcomes [24]. To date, three $\alpha 2$ adrenergic receptors have been described: $\alpha 2A$, $\alpha 2B$ and $\alpha 2C$. All of these receptors work along with G protein inhibitor and play an important role in controlling pain perception. In this context, an increase in the $\alpha 2A$ receptors has been shown in various experimental interventions that have resulted in pain relief [25]. Experimental studies have demonstrated a significant relief of neuropathic pain due to diabetes following the intraperitoneal or systemic administration of $\alpha 2$ adrenergic agonists [25]. In addition, clinical studies have reported that they produced significant pain relief in patients suffering from severe diabetic neuropathy or cancers [25]. The beneficial effects of exercise are likely to come from the vasculogenic, neurotrophic and antioxidant capacities. Although we did not examine the Brain-Derived Neurotrophic Factor (BDNF) due to exercise in the current study, earlier studies have shown its positive effects on the $\alpha 2$ adrenergic receptors, pain control and raising the levels of neurotrophic factors following exercise [26]. The findings of the current study indirectly support the beneficial effect of exercising on $\alpha 2$ adrenergic receptors and potentially on BDNF.

To date, the effects of using nano-eugenol supplements combined with exercise on the levels of inflammatory factors in DRG region have not been investigated. However, it has been demonstrated that eugenol has considerable anti-inflammatory properties and its consumption has relieved pain of neuropathic origin. This beneficial effect is likely due to the nano-eugenol ability to inhibit oxidative stressors and to repair the resultant neuronal damages [27]. It seems that combining physical exercise with nano-eugenol supplementation significantly enhances the anti-inflammatory effects and minimizes the resultant cellular damages in DRG region. This concept is consistent with our earlier histologic observations [28] from the DRG region of the rats used in a previous study, where diabetes reduced the population of DRG neurons while an 8-week exercise protocol increased them. A similar outcome was observed with nano-eugenol administration alone. Interestingly, combining exercise with nano-eugenol significantly enhanced the therapeutic effect and brought the DRG cell numbers close to those in the normal controls. Finally, it may be concluded that using nano-eugenol together with regular, moderate intensity aerobic exercises significantly minimizes the associated nervous damages in diabetes, likely by in-

hibiting the release or synthesis of inflammatory factors, especially P38-MAPK in the DRG region.

Conclusions

Based on the findings of the current study, the following conclusions may be made:

Aerobic exercising or nano-eugenol supplementation alone is effective in controlling damages to DRG neurons, likely caused by inflammatory factors, such as P38-MAPK, raised in diabetes.

Aerobic exercise combined with nano-eugenol supplementation is significantly more effective in controlling damages to the DRG neurons caused by P38-MAPK, NPY and A-R $\alpha 2A$ prevalent in diabetes than either approach alone.

The levels of the above inflammatory factors are most likely associated with damages to the ganglionic cells in DRG region. These factors potentially contribute to the neuropathic pain in diabetics.

Aerobic exercise combined with nano-eugenol may negatively regulate the synthesis and/or release of the above inflammatory factors in the CNS, particularly in the DRG region.

Limitations of the study: In the current study, changes in other endogenous anti-inflammatory mediators were not investigated; however, it is likely that the release and/or activation of other anti-inflammatory factors have influenced the study's outcomes.

Recommendations for future studies: We recommend that future studies on this subject should use a larger sample size of rats to investigate P38-MAPK, NPY and A-R $\alpha 2A$ and/or other inflammatory and anti-inflammatory mediators versus different exercise protocols at varying intensity and duration.

Ethical Considerations

Compliance with ethical guidelines

The study protocol was approved by the Research Ethics Committee, Islamic Azad University, Khorasgan Branch, Isfahan (Ethics Code #: IR.IAU.KHUISE.REC.1399.046).

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Author's contributions

Conceptualization, supervision: Abbas Shareghi Boroujeni & Khosro Jalali Dehkordi; Methodology: Abbas Shareghi Boroujeni; Investigation, Writing – original draft, and Writing – review & editing: All authors.

Conflict of interest

The authors declare no conflict of interests with any internal or external entity in conducting this research.

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