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Antinociceptive effects of gamma-linolenic acid in the formalin test in the rats

Kaveh Rahimi, PhD[a,*], Arman Nourishirazi[a], Hamidreza Delaviz[a], Zohreh Ghotbeddin, PhD[a,b]

Background: Gamma-linolenic acid (GLA) is found in animals and plants that play a role in brain function and metabolism.
Objective: This study aimed to investigate the analgesic effects of GLA on peripheral formalin injection.
Methods: Wistar rats were randomly assigned to four groups: Sham, formalin, formalin/GLA 100 mg/kg, and formalin/GLA 150 mg/kg. The Formalin test was utilized to create a pain model. A tissue sample was prepared from the spinal cords of rats to measure oxidative stress parameters and pro-inflammatory cytokines. Furthermore, the authors analyzed the expression of c-Fos protein in the spinal cords.
Results: Our findings demonstrate that GLA has a reliable pain-relieving effect in the formalin test. GLA 100 increased superoxide dismutase (SOD) ($P < 0.05$), glutathione (GSH) ($P < 0.001$), and catalase (CAT) ($P < 0.05$), and decreased the levels of c-Fos ($P < 0.001$), interleukin-1 beta (IL-1β) ($P < 0.001$), tumour necrosis factor-alpha (TNF-α) ($P < 0.001$), and malondialdehyde (MDA) ($P < 0.001$) in the spinal cord. Also GLA 150 increased SOD ($P < 0.05$), GSH ($P < 0.001$), and CAT ($P < 0.05$) and decreased the levels of c-Fos ($P < 0.001$), IL-1β ($P < 0.001$), TNF-α ($P < 0.001$), and MDA ($P < 0.001$) in the spinal cord.
Conclusion: The findings have validated the antinociceptive impact of GLA and hinted towards its immunomodulatory influence in the formalin test.

Keywords: c-Fos, formalin, gamma-linolenic acid, inflammation, oxidative stress

Introduction

Pain can result from tissue damage, chemicals, or abnormal immune system activation that triggers pain receptors. It is widely recognized that the spinal cord plays a crucial role in transmitting pain signals from damaged tissues. Chronic conditions, such as inflammation, can change the characteristics of the somatic sensory pathways. This can lead to symptoms such as hyperalgesia, which is an increased sensitivity to pain. It can also cause changes in the excitability of primary afferent neurons, which are responsible for transmitting sensory information from the body to the brain[1–3]. Following the injection of irritant chemicals such as formalin into the peripheral region, cell migration, oedema, and fever may be induced. This process is mediated by cytokines and prostaglandins. Additionally, pro-inflammatory mediators have the potential to sensitize pain afferent neurons and increase pain behaviour, resulting in hyperalgesia or allodynia[4]. The formalin test was created by Dubuisson and Dennis in 1977 to evaluate pain-related reactions and has been widely used since[5]. Various animal species, such as rats, mice, rabbits, cats, guinea pigs, and primates, are used[6].

The c-Fos gene encodes the nuclear protein Fos and is rapidly and transiently expressed in neurons in response to a pain stimulus[7]. FOS can alter spinal pain circuits, leading to modulation of spinal pain processes[8–10].

According to research, the spinal cord microglia's become reactive before the astrocytes when inflammation occurs in the body. In situations of inflammatory pain, the dorsal horn of the spinal cord produces pro-inflammatory cytokines due to peripheral macrophages and activated microglia[11–14]. As a result, these cells increase the expression of cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6), which are associated with inflammation-induced pain[15].

HIGHLIGHTS

- Gamma-linolenic acid (GLA) has a reliable pain-relieving effect in the formalin test.
- GLA increased superoxide dismutase (SOD), glutathione (GSH), and catalase (CAT) and decreased the levels of c-Fos, interleukin-1 beta (IL-1β), tumour necrosis factor-alpha (TNF-α), and malondialdehyde (MDA) in the spinal cord.
- The findings have validated the antinociceptive impact of GLA and hinted towards its immunomodulatory influence in the formalin test.

[a]Department of Basic Sciences, Faculty of Veterinary Medicine and [b]Stem Cell and Transgenic Technology Research Center, Shahid Chamran University of Ahvaz, Ahvaz, Iran

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*Corresponding author. Address: Assistant Professor, Department of Basic Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran. Tel.: 98613330073; fax: 98613360807. E-mail: k.rahimi@scu.ac.ir. Kaveh_rahimi66a@yahoo.com (K. Rahimi).

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The increase of IL-1β and TNF-α can lead to the induction of oxidative stress[16].

The polyunsaturated fatty acids (PUFAs) are important for both health and disease. The body produces powerful modulatory molecules for inflammatory responses, such as prostaglandins, leukotrienes, and interleukins. These molecules affect the gene expression of various bioactive molecules. Gamma-linolenic acid (GLA), which is an essential fatty acid of the omega-6 series, is produced by the body from linoleic acid through the delta-6-desaturase enzyme. Linoleic acid is also known as all cis 6, 9-octadecadienoic acid. While the positive effects of GLA supplementation have been established, the exact molecular mechanism responsible for its health benefits remains unclear[17]. There are some studies that have pointed to the analgesic effects of GLA. GLA may be helpful in managing breast pain and reducing nerve pain in people with diabetic neuropathy[18]. Pretreatment with GLA in diabetic rats reduced the pain perception caused by the tail-flick test[19]. GLA reduces inflammation by deactivating nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) through the reduction of oxidative stress and inhibition of the inflammatory pathways[20]. It is imperative to conduct studies on the possible mechanisms behind GLA's pain-relieving properties to complete its analgesic profile. The current lack of information on this topic needs to be addressed. It has previously been demonstrated that injecting formalin peripherally can increase the levels of inflammatory mediators and oxidative stress in the spinal cord[21]. Our goal was to study the effects of GLA on pain responses caused by peripheral formalin administration. Additionally, we researched the molecular mechanisms contributing to GLA's pharmacological properties.

Methods

Animals

Thirty two male Wistar rats, with an average weight of 250 ± 20 g, were housed in a facility with a 12-h light and 12-h dark cycle, at a temperature of 22 ± 2°C. They were given free access to food and water. The experiments were carried out according to the ARRIVE (Animal Research: Reporting of in Vivo Experiments) guidelines 2.0[22]. GLA was derived from evening primrose oil produced by Barij Essence Pharmaceutical Company in Iran. The protocol of this study was approved by the Research Ethics Committee (EE/1401.2.24.14812/scu.ac.ir).

Study design

There were four groups of animals in this study ($n = 8$). The first group received sunflower oil for seven days. They also received a subcutaneous injection of normal saline on the right hind paw on the seventh day. The second group received a formalin injection of 50 µl with a concentration of 2.5% in their right hind paw after receiving sunflower oil injection for seven days. The third group, known as the GLA 100 group, was injected with GLA 100 mg/kg of sunflower oil for seven days before undergoing the formalin test. Lastly, the GLA 150 group was injected with GLA 150 mg/kg of sunflower oil for seven days before the formalin test was conducted[23]. It is important to note that both the drug and drug carrier were administered through an intraperitoneal injection.

Formalin test

Prior to the experiment, rats were placed in a test chamber for 20 min to become accustomed to their surroundings. Then, a 50 µl injection of 2.5% formalin solution (dissolved in NaCl 0.09%) was administered subcutaneously into the rat's right hind paw using a syringe[24,25]. The behavioural data was recorded for 40 min following the formalin injection. The Formalin test is divided into two phases: acute phase (0–5 min) and chronic phase (16–40 min)[5,26].

Tissue sampling

The rats were anesthetized using a combination of ketamine and xylazine (40 and 5 mg/kg, receptively) and immediately (< 60 sec) sacrificed[27]. After injecting formalin, samples were collected from the spinal cord of four animals in each group to assess biochemical parameters 24 h later[28]. In each group, the L4–6 spinal cords were isolated to evaluate c-Fos protein levels 2 h after formalin injection ($n = 4$)[29,30]. All the samples were stored at – 70°C until further testing.

Measurement of biochemical parameters

Samples of the spinal cords were taken to analyze various biochemical parameters such as malondialdehyde (MDA), IL-1β, and TNF-α. The antioxidant parameters such as glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) were also analyzed. The total protein content was measured in all samples using the Bradford method. These parameters were established using the Enzyme-Linked Immunosorbent Assay technique (ELISA). All these analyses were done using Kiazist products from Hamedan, Iran, with the following product numbers: KMDA96 (MDA), E0119Ra (IL-1β), E0764Ra (TNF-α), KTHI96 (GSH), KSOD96 (SOD), KCAT96 (CAT), and KBRD96 (total protein content).

Western blot

Tissues were lysed using a buffer and a protease inhibitor. Samples were homogenized and centrifuged. Protein concentration was measured, and samples were boiled and transferred to a gel for electrophoretic separation and immunoblotting. Band density was calculated by scanning photosensitive papers. Protein density was analyzed using JS 2000 software.

Statistical analyses

We utilized version 26 of SPSS software to analyze the data. Before proceeding, we conducted a normalization test on the data based on their distribution and homogeneity of variances, using the Kolmogorov–Smirnov test available in SPSS. The test revealed that the data were normally distributed, so we performed a one-way ANOVA to assess the groups. Post hoc analyses were conducted using the Tukey test. A significance level of P less than 0.05, P less than 0.01, and P less than 0.001 were considered.

Results

Formalin test

Figure 1 shows the results of the formalin test (mean ± ESM). During the acute phase (0–5 min) of the test, the formalin group had higher average pain intensity than the sham group

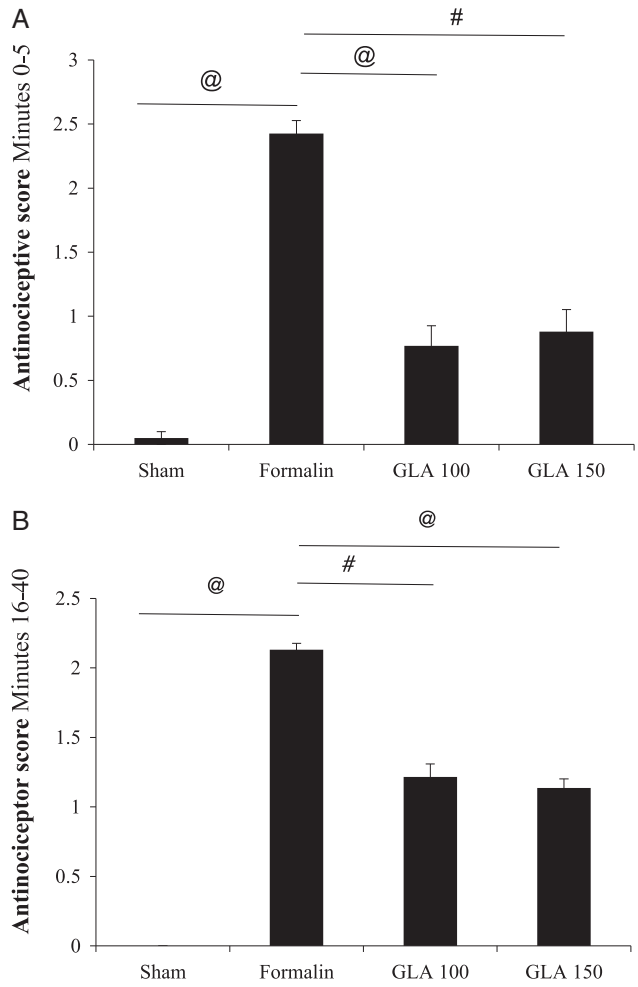

Figure 1. Effect of GLA pre-treatment (100 and 150 mg/kg) on pain behaviour in the formalin test. (A) Antinociceptor score between 0 and 5 min. (B) Antinociceptor score between 16 and 40 min. All data are presented as the mean ± SEM (n = 8 per group). One-way ANOVA was used to identify the overall differences between the groups. The significant statistical difference between the specified groups revealed by post hoc Tukey's test. *$P <$ 0.05, # $P <$ 0.01 and @ $P <$ 0.001. GLA, Gamma-linolenic acid.

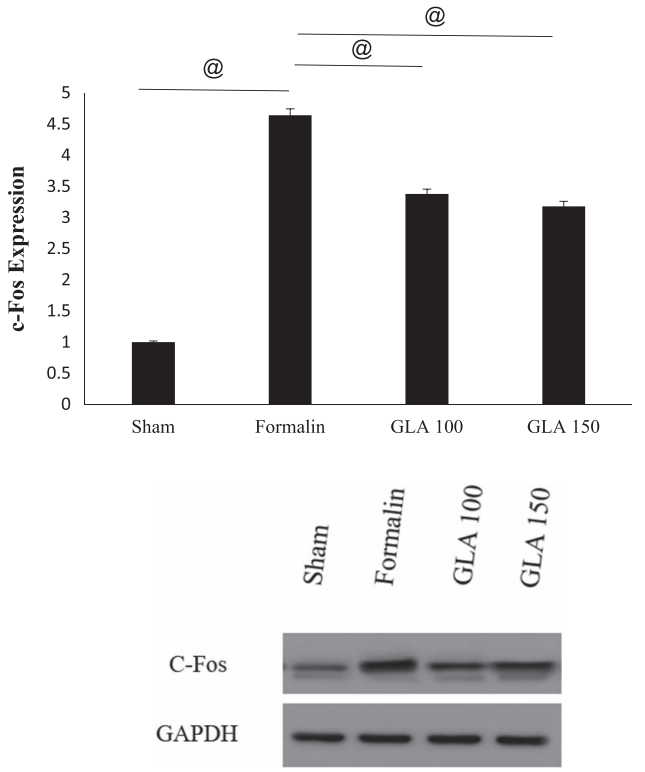

Figure 2. Effect of GLA pre-treatment (100 and 150 mg/kg) on c-Fos protein expression (western. blot). All data are presented as the mean ± SEM (n = 4 per group). One-way ANOVA was used to identify the overall differences between the groups. The significant statistical difference between the specified groups revealed by post hoc Tukey's test. @ $P <$ 0.001. GLA, Gamma-linolenic acid.

($P < 0.001$). However, groups GLA 100 and 150 showed lower average pain intensity than the formalin group ($P < 0.001$, $P < 0.01$). During the chronic phase (16–40 min), the formalin group showed higher pain intensity compared to the sham group ($P < 0.001$). Additionally, groups GLA 100 and 150 exhibited lower average pain intensity than the control group ($P < 0.01$, $P < 0.001$).

The c-Fos protein expression in the spinal cord

It was observed that there was an increase in the amount of c-Fos protein in the spinal cord after formalin injection as compared to the sham group ($P < 0.001$). Additionally, we conducted an analysis to determine the effects of GLA on the expression of c-Fos in the spinal cord in the formalin test. The results showed a difference between the formalin group and the GLA 100 ($P < 0.001$) as well as the 150 mg/kg ($P < 0.001$) groups (Fig. 2).

The levels of IL-1β in the spinal cord

Following the injection of formalin, it was observed that the formalin group had higher levels of IL-1β compared to the sham group ($P < 0.001$). However, by administering GLA 100 and 150 mg/kg, the IL-1β levels in the formalin group were reduced ($P < 0.001$, $P < 0.001$), as demonstrated in Fig. 3.

The levels of TNF-α in the spinal cord

After injecting formalin, the TNF-α levels were found to be higher in the formalin group in comparison to the sham group ($P < 0.001$). However, when GLA was administered at a dose of 100 and 150 mg/kg, the TNF-α levels decreased as compared to the control group ($P < 0.001$, $P < 0.001$), as shown in Fig. 3.

The levels of SOD, GSH, CAT, and MDA in the spinal cord

Our study showed that animals treated with formalin had lower levels of SOD, GSH, and CAT in their spinal cord compared to healthy rats ($P < 0.001$, $P < 0.001$, $P < 0.001$). However, administering GLA at dosages of 100 mg/kg resulted in increased levels of SOD, GSH, and CAT compared to the formalin group ($P < 0.05$, $P < 0.001$, $P < 0.05$). In addition, GLA at a dosage of 150 mg/kg significantly increased SOD, GSH, and CAT levels compared to the formalin group ($P < 0.05$, $P < 0.001$, $P < 0.05$). Furthermore, we observed increased levels of MDA in the spinal cord of animals treated with formalin compared to healthy rats

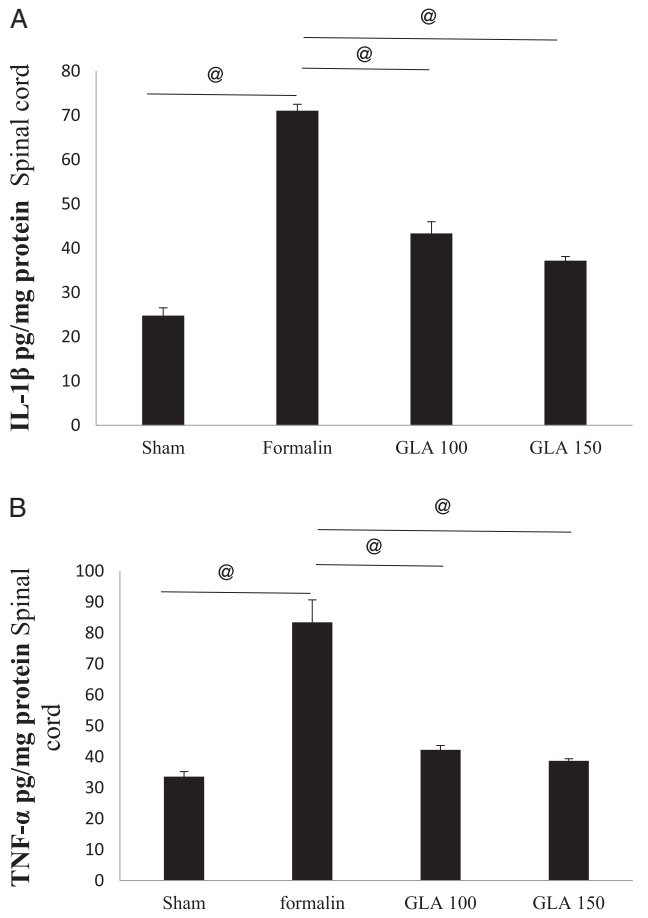

Figure 3. Effect of GLA pre-treatment (100 and 150 mg/kg) on IL-1β and TNF-α levels. (A) The. levels of IL-1β in the spinal cord. (B) The levels of TNF-α in the spinal cord. All data are presented as the mean ± SEM (n = 4 per group). One-way ANOVA was used to identify the overall differences between the groups. The significant statistical difference between the specified groups revealed by post hoc Tukey's test. *P < 0.05, #P < 0.01 and @P < 0.001. GLA, Gamma-linolenic acid.

($P < 0.001$). Nevertheless, administering GLA at dosages of 100 and 150 mg/kg resulted in decreased levels of MDA compared to the formalin group ($P < 0.001$, $P < 0.001$) (Fig. 4).

Discussion

GLA is an 18C omega-6 polyunsaturated fatty acid in black-currant, borage, evening primrose oil, and milk[31]. Current studies on GLA primarily focus on its potential therapeutic benefits for inflammation[32]. In various clinical studies, GLA adjuvant has been studied as a potential treatment for influenza, tuberculosis, malaria, HIV, schistosomiasis, leishmaniasis, and Hansen's disease[33]. Also, changes in blood GLA levels have been reported in some diseases[34]. In our search, we found only a few studies that describe the use of GLA for pain[17,20]. In diabetic rats, the GLA decreased the pain perception caused by the tail-flick test[19]. Furthermore, studies have demonstrated that GLA can effectively decrease breast pain[18]. Despite the potential benefits of using PUFAs, such as GLA, there are limitations when it comes to conducting large clinical trials. This is due to the fact that subjects may have varying levels of 18C- and LC-PUFA in their circulation and tissue, resulting in inconsistent reactions to PUFA-based supplements. As such, further studies are needed to fully understand the effects of these supplements. Balancing n-6 and n-3 metabolic pathways can improve health by reducing inflammation and preventing disease. PUFA-based supplements, such as GLA-enriched supplements, can be used in complex supplementation strategies to address individual and population differences for personalized medicine[35]. In our study, we aimed to minimize these variables by using rats of the same breed and sex.

In the present study, both GLA 100 and a dosage of 150 mg/kg were effective in reducing the pain intensity in the formalin test. It is worth noting that there was no significant difference observed between the 100 mg/kg and 150 mg/kg dose of GLA. In the acute phase of the formalin test, the group that received formalin treatment experienced higher average pain intensity than the normal rat group. However, the groups treated with GLA 100 and 150 mg/kg showed a decrease in pain intensity compared to the formalin group[6]. During the second phase of the formalin test, research has shown that the pain experienced is due to the central sensitization of spinal dorsal horn neurons[6]. The sensitivity of central neurons can be verified by an increase in the c-Fos[36].

The C-Fos expression in the spinal cord increased 2 h after formalin injection[37–39]. The c-Fos gene is an immediate-early gene that is rapidly transcribed into messenger RNA, leading to the expression of Fos protein that lasts for up to an hour[40]. In the present study, treatment with GLA 100 and 150 mg/kg reduced c-Fos expression in the spinal cord following formalin injection. A study has reported that mRNA for c-Fos is reduced markedly in human T cells incubated with GLA in patients with rheumatoid arthritis. In that study, the reduction of c-Fos interacted with the reduction of IL-2[41].

The injection of formalin into the periphery caused elevated levels of IL-1β and TNF-α in the spinal cord. When the inflammasome is activated, certain molecules are released as a response by the body. This leads to an increase in pro-inflammatory cytokines. These cytokines can be transported in a retrograde manner either through axonal or non-axonal mechanisms from the environment to the spinal cord. This can contribute to nociception[42]. Based on our research, after formalin administration, GLA was found to have neuroimmunomodulatory effects on the spinal cord by reducing concentrations of IL-1β and TNF. Neuroinflammation is an important factor in nociceptive transmission at both the spinal and supraspinal levels[17]. It has been reported that compounds containing PUFAs, including fish oil, have antinociceptive effects[43]. GLA inhibits inflammatory responses by suppressing ERK/JNK signal transduction and oxidative stress, leading to NF-kB inactivation[20]. Inactivation of the NF-κB pathway suppresses the production of IL-6 and TNF-α[44].

Previous studies have shown that TNF-α and IL-1β stimulate the biogenesis of cellular ROS in various tissues, resulting in their generation[45,46]. Oxidative stress plays a crucial role in inflammatory responses. The process of oxidative stress can cause damage to proteins, lipids, and DNA in both neurons and glial cells[47]. This can result in a loss of function due to oxidative degradation. Our study has shown that when formalin is injected into the peripheral system, it leads to elevated levels of oxidative stress markers like MDA in the spinal cord. On the other hand, the production of antioxidant markers such as SOD, CAT, and GSH is impaired. However, injection of GLA 100 and 150 mg/kg

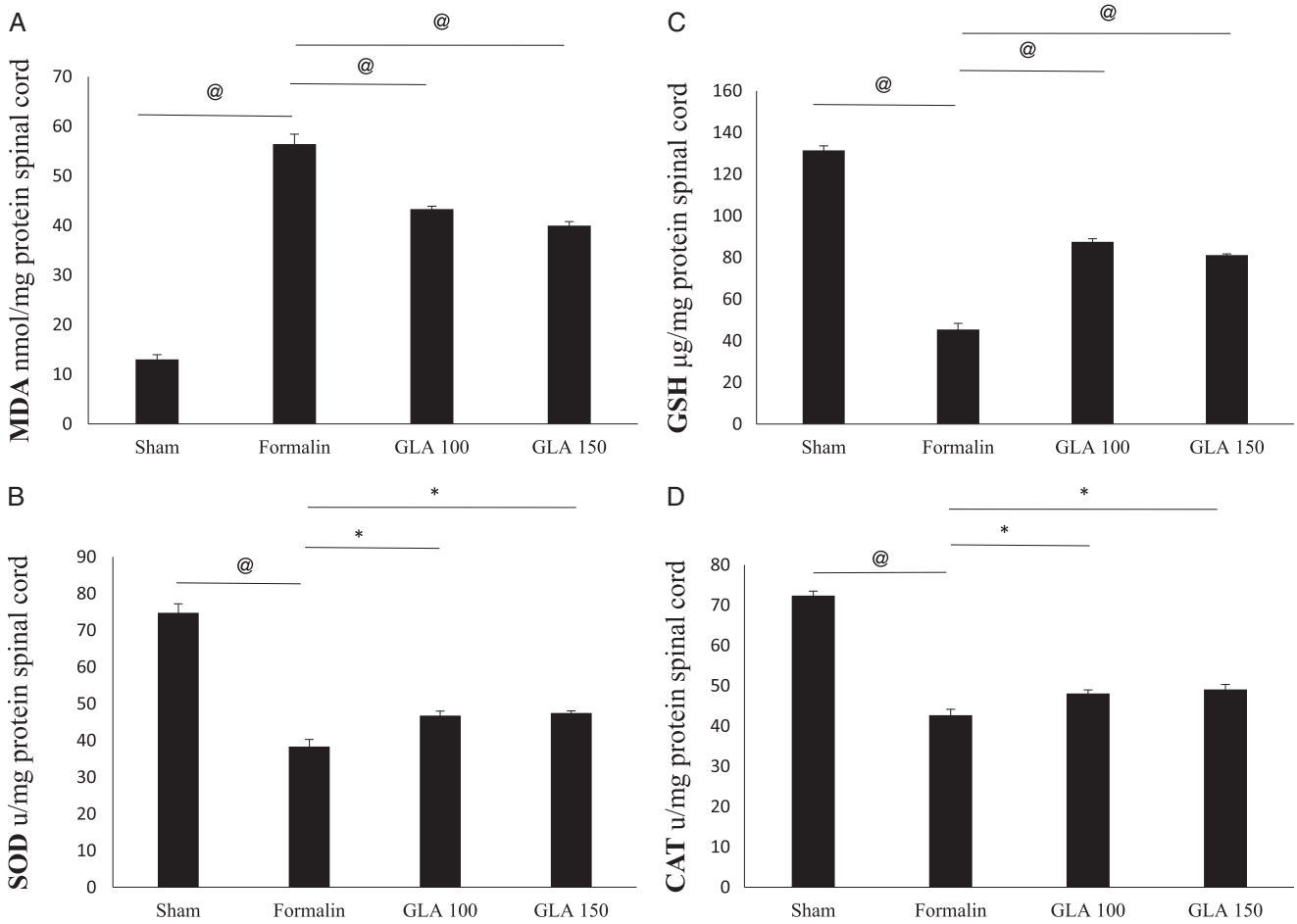

Figure 4. Effect of GLA pre-treatment (100 and 150 mg/kg) on (A) MDA, (B) SOD, (C) CAT, and (D) levels in the spinal cord. All data are presented as the mean ± SEM ($n = 4$ per group). One-way ANOVA was used to identify the overall differences between the groups. The significant statistical difference between the specified groups revealed by post hoc Tukey's test. *$P < 0.05$, # $P < 0.01$ and @ $P < 0.001$. GLA, Gamma-linolenic acid.

decreased MDA levels and increased SOD, CAT, and GSH in the spinal cord compared with the formalin-treated group. Previous studies have suggested that the analgesic effects of GLA are mediated by the neuroprotective, vasodilatory, and antioxidant properties of GLA[48].

Conclusion

This study shows that GLA has anti-hyperalgesic effects in formalin tests. The immunomodulatory mechanism of GLA has been partially explained, indicating that it has an impact on the spinal cord and inflammation site. According to the study, GLA can reduce the levels of pro-inflammatory cytokines like IL-1β and TNF-α in the spinal cord. This indicates that it may have a neuroimmunomodulatory impact on central sensitization. Furthermore, the administration of GLA resulted in a significant alteration of oxidative stress markers in the spinal cord. In individuals who experience pain, such as those with rheumatoid arthritis, supplementation with GLA may be beneficial[49]. However, further studies are needed in order to better comprehend the mechanism behind GLA's pain-relieving effects.

Ethical approval

The protocol of this study was approved by the Research Ethics Committee of Shahid Chamran University of Ahvaz, Ahvaz, Iran (EE/1401.2.24.14812/scu.ac.ir). 1/2/2023.

Consent

The experiments were performed in compliance with the Animal Research: Reporting of in vivo Experiments guidelines. The present study involved client-owned animals and demonstrated a high standard (best practice) of veterinary care and involved informed client consent.

Source of funding

Shahid Chamran University of Ahvaz, Iran.

Author contribution

K.R., A.N., H.D., and Z.G. conceived and designed the project. K.R., A.N., H.D., and Z.G. collected the data. K.R. analyzed and

interpreted the data. K.R., A.N., H.D. and Z.G. drafted the manuscript. All authors read and approved the final manuscript."

Conflicts of interest disclosure

The authors declare that they have no competing interest.

Research registration unique identifying number (UIN)

Our research was animal study (EE/1401.2.24.14812/scu.ac.ir).

Guarantor

Kaveh Rahimi is the person in charge of the publication of our manuscript.

Data availability statement

Data sharing is not applicable to this article.

Provenance and peer review

The authors confirm that this study not commissioned, externally peer-reviewed.

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