

# Research Paper: The Anti-nociceptive Effect of Ellagic Acid in Streptozotocin-induced Hyperglycemic Rats by Oxidative Stress Involvement



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## ABSTRACT

**Introduction:** Hyperalgesia is among the current complications of diabetes mellitus; oxidative stress and inflammation were influential in its development. As an herbal component, Ellagic Acid (EA) has some biological activities, including antioxidant and anti-inflammatory effects. This study was designed to evaluate the possible beneficial effect of EA on hypernociception in Streptozotocin (STZ)-induced hyperglycemic rats.

**Methods:** Forty-eight male Wistar rats were divided into the control (receiving vehicle), hyperglycemic, EA (25 mg/kg)-treated control, EA (50 mg/kg)-treated control, EA (25 mg/kg)-treated hyperglycemic, and EA (50 mg/kg)-treated hyperglycemic groups. Hyperglycemia was induced by a single Intraperitoneal (IP) injection of STZ (60 mg/Kg). EA was administered daily by oral gavage for four weeks. The nociceptive response was assessed using Tail-Flick (TF) and Hot-Plate (HP) tests. Also, oxidative stress markers, including Malondialdehyde (MDA), Total Oxidant Status (TOS), and Total Antioxidant Capacity (TAC) in the serum, were evaluated.

**Results:** Hyperglycemic animals were found with significant changes, including a reduction in TF and HP latencies, an elevation in serum MDA level and TOS, and a decrease in serum TAC compared with controls. The treatment of hyperglycemic rats with EA facilitated the reduction of TF latency at the dose of 25 mg/kg and HP latency at 50 mg/kg. Furthermore, EA significantly increased TAC and decreased MDA level at a 50 mg/kg dose and reduced TOS at both doses in the serum of hyperglycemic animals. No significant alterations were found in the parameters studied in EA-treated normal rats.

**Conclusion:** These results displayed the antinociceptive effect of EA in hyperglycemic rats via attenuating oxidative stress. Therefore, EA appears to be a promising agent for managing. Hyperglycemic hypernociception.

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## Highlights

- Hyperalgesia is among the current complications of diabetes mellitus.
- Oxidative stress and inflammation were influential in its development.
- EA has some biological activities, including antioxidant and anti-inflammatory effects.

## Plain Language Summary

DN is among the most common chronic complications of diabetes among diabetic patients. DPN is mainly characterized by pain perception alterations, increased sensitivity to mild painful stimuli (hyperalgesia), and abnormal pain sensitivity to stimuli. On the other hand, hyperglycemia by induction of multiple changes, such as fatty acid metabolism abnormalities plays a crucial role in developing DN. Oxidative stress and inflammation are involved in the pathogenesis of DPN. The lack of efficacy and various adverse effects of the current medications for DNP, therefore, new therapeutic candidates are continuously required to improve DNP. Several studies reported the antinociceptive activity of EA in different animal models of pain, such as formalin. Since oxidative stress is involved in diabetic hyperalgesia, compounds with antioxidant properties are good candidates for DN management. Therefore, this research aimed to determine the possible effectiveness of EA and evaluate some oxidative stress-related mechanisms.

### 1. Introduction

**D**iabetic Neuropathy (DN) is among the most common chronic complications of diabetes that develop in >60% of diabetic patients (Cameron & Cotter, 2008). Diabetic Painful Neuropathy (DPN) is mainly characterized by pain perception alterations, increased sensitivity to mild painful stimuli (hyperalgesia), and abnormal pain sensitivity to stimuli, i.e., not previously painful (Hasanein & Fazeli, 2014). The persistent sensation of burning, aching, or spontaneous pain significantly affects the patient's life quality (Veves, Backonja, & Malik, 2008). Hyperglycemia by induction of multiple changes, such as fatty acid metabolism abnormalities and advanced glycation in peripheral neurons and glia and vascular cells, plays a crucial role in developing and progressing DN (Dobretsov, Hastings, Stimers, & Zhang, 2001).

Oxidative stress and inflammation are crucial pathways involved in the pathogenesis of DPN (Green, Pedersen, Pedersen, & Scheele, 2011; Rodrigues, Bergamaschi, Araújo, Mouro, Rosa, & Higa, 2011). Advanced Glycation End (AGE) products stimulate the production of Reactive Oxygen Species (ROS) and the expression of pro-inflammatory cytokines, such as Interleukin-1 (IL-1) and Tumor Necrosis Factor- $\alpha$  (TNF- $\alpha$ ) (Kuhad, Bishnoi, & Chopra, 2009). In diabetic rats, the overproduction of ROS and the activation of Lipid Peroxidation (LPO) were observed in the sciatic nerve (Cunha, Jolival, Ramos, Gregory, Calcutt, & Mizisin, 2008). Addition-

ally, reduced pain threshold and attenuated antioxidant enzyme activity were reported in the sciatic nerve of diabetic rats (Al-Enazi, 2013). Hyperglycemia-induced oxidative stress in diabetic patients may cause spontaneous impulses in pain afferent neurons and spinothalamic neurons by inducing hypersensitivity in these neurons (Chen & Pan, 2002; Khan, Chen., & Pan, 2002). The lack of efficacy and various adverse effects of the current medications for DNP, such as tricyclic antidepressants, anticonvulsants, topical anesthetics, and opioids, have limited their usage (Hasanein & Fazeli, 2014). Therefore, new therapeutic candidates are continuously required to improve DNP.

Ellagic Acid (EA) is a polyphenol compound found in numerous vegetables, fruits, and nuts, such as tomatoes, carrots, strawberries, raspberries, and pomegranates. Furthermore, EA exhibits different pharmacological properties, including antioxidant, anti-inflammatory, anti-apoptotic, and anti-cancer effects (Uzar et al., 2012). EA can reduce oxidative damage via scavenging free radicals, increasing antioxidant enzyme activity, and decreasing LPO (Chao, Hsu, & Yin, 2009; Uzar et al., 2012; Kiasalari et al., 2017). This compound was found to decrease oxidative damage and reduce the levels of Malondialdehyde (MDA) and Total Oxidant Status (TOS) in the sciatic nerve and brain of diabetic rats (Uzar et al., 2012). EA possesses anti-inflammatory properties, such as reducing IL-6 and TNF- $\alpha$ , blocking the NF- $\kappa$ B pathway, inhibiting COX-2, and increasing IL-10 (Cornélio Favarin et al., 2013; Kaur, Mehan, Khanna, & Kalra,

2015). Several studies reported the antinociceptive activity of EA in different animal models of pain, such as formalin, acetic acid, and carrageenan-induced hyperalgesia (Taghi Mansouri, Naghizadeh, Ghorbanzadeh, & Farbood, 2013).

Since oxidative stress is involved in diabetic hyperalgesia, compounds with antioxidant properties are good candidates for DN management. Therefore, this research was conducted to determine the possible effectiveness of EA, as a free radical scavenger, on hypernociception in hyperglycemic rats and evaluate some oxidative stress-related mechanisms.

## 2. Methods

Forty-eight adult male Wistar rats (9-11 weeks old, 230-250 g) were obtained from the Hamadan University of Medical Sciences animal house. The animals were maintained under the controlled conditions (50%±5% humidity and 20±2 °C temperature) under a 12:12 h light/dark cycle with free access to water and food.

The animal care and treatment procedures were approved by the Ethics Committee of the Hamadan University of Medical Sciences (Code: IR.UMSHA.REC.1395.284).

The rats were randomly divided into 6 groups (n=8) as follows:

Group 1: Normal control rats receiving solvent for 28 days.

Groups 2: Control rats receiving EA (25 mg/kg/day) for 28 days.

Groups 3: Control rats receiving EA (50 mg/kg/day) for 28 days.

Group 4: Hyperglycemic rats receiving solvent for 28 days.

Groups 5: Hyperglycemic rats receiving EA (25 mg/kg/day) for 28 days.

Groups 6: Hyperglycemic rats receiving EA (50 mg/kg/day) for 28 days.

Hyperglycemia was induced by a single Intraperitoneal (IP) injection of 60 mg/kg of STZ (Santa Cruz, USA). Three days after STZ injection, Fasting Blood Glucose (FBS) level was measured (Deeds et al., 2011; Zarrinkalam et al., 2018). Rats with an FBS level over 250 mg/

dL were considered hyperglycemic. Then, the administration of EA (Sigma Aldrich, USA) (25 & 50 mg/kg) or solvent was started once daily for 4 weeks by oral gavage. The used doses of EA were selected based on a previous report (Kaur et al., 2015) and our earlier pilot study. Normal saline containing 10% DMSO was used as the solvent of EA.

Pain-related behaviors measurable by the Tail-Flick (TF) and Hot-Plate (HP) tests were evaluated at the end of the treatments (24 h after the last EA treatment).

### Tail-flick test

The nociceptive response was estimated using a TF apparatus (BorjSanat, Tehran, Iran) according to the method proposed by D'Amour and Smith (D'Amour & Smith, 1941). Briefly, the dorsal surface of the rat tail was exposed to a beam of light as a radiant heat source and the time required to flick the tail from the thermal stimulus was recorded. The cut-off time was set at 10 s to prevent tail tissue injury.

### Hot-plate test

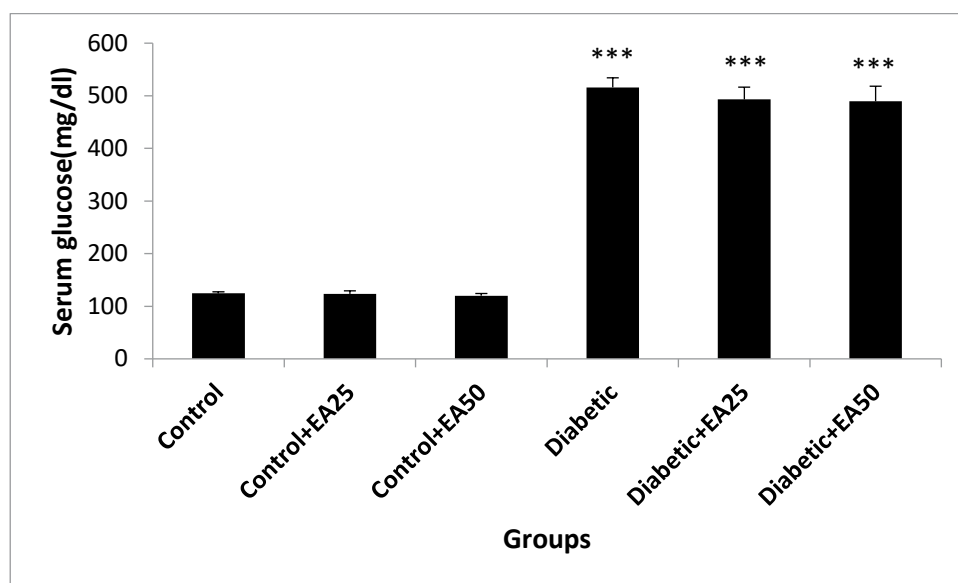
Animals were put individually on a hot-plate analgesia meter (BorjSanat, Tehran, Iran) with a temperature adjusted to 55±0.1 °C. The latency of behaviors like jumping, hind paw licking, or hind paw flicking was recorded as the pain response. A cut-off time of 35 s was established to avoid tissue damage (Shirafkan, Sarihi, & Komaki, 2013).

### Biochemical analysis

At the end of the experiment, body weight was measured. Then, the blood sample was drawn under anesthesia from inferior vena cava for both plasma glucose measurement using a kit (Zistshimi, Tehran, Iran) and the estimation of oxidative stress biomarkers.

The MDA level in the serum as the end product of LPO was measured by the thiobarbituric acid reactive substance assay as described before (Kamal, Gomaa, El Khafif, & Hammad, 1989). Briefly, trichloroacetic acid and thiobarbituric acid reagent were added to the serum, and then the mixture was heated in a boiling water bath for 45 min. The following cooling on ice and mixing with n-butanol, the sample was centrifuged, and the absorbance was read at 532 nm. The obtained results were expressed using the tetraethoxypropane standard curve.

TAC in the serum was assessed by evaluating the ability to decrease Fe<sup>3+</sup> to Fe<sup>2+</sup>, applying the Ferric Reduc-



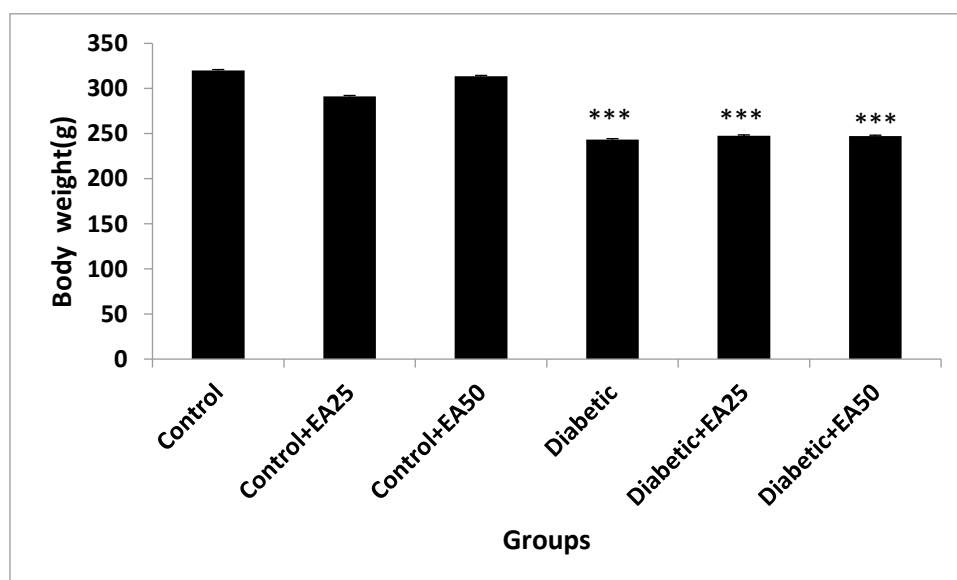
**Figure 1.** Effect of Ellagic Acid (EA) treatment on body weight

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Data are represented as Mean $\pm$ SEM. \*\*\*P<0.001 compared with the control group. Statistical analysis was performed using one-way ANOVA followed by Tukey's Post-Hoc Test

ing Antioxidant Power (FRAP) test. The medium was exposed to  $\text{Fe}^{3+}$ , and then  $\text{Fe}^{2+}$  production, as antioxidant activity, was noticed. In short, acetate buffer and 4, 6 Tripyridyl-s-Triazine (TPTZ) were mixed with standards, and the samples at 37°C. Reagent that was not added to the standard or sample was applied as a blank. The absorbance measurement was performed at 593nm (Pohanka et al., 2009).

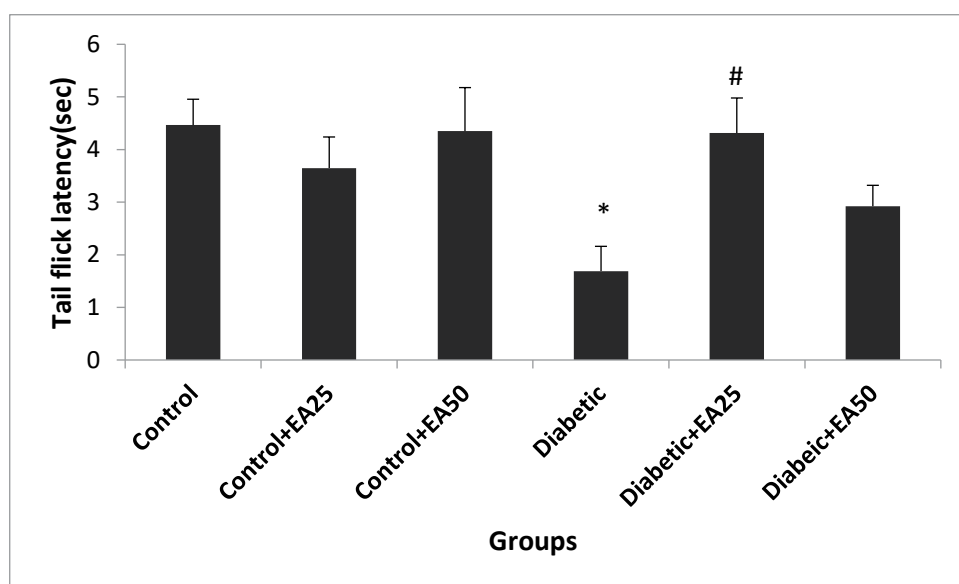
TOS was measured in the serum using the Erel method, by which oxidation of  $\text{Fe}^{2+}$  to  $\text{Fe}^{3+}$  by oxidants found in the sample was determined. The color intensity that  $\text{Fe}^{3+}$  forms with xylenol orange in an acidic medium were related to the number of oxidant molecules of the sample. This intensity was measured using a spectrophotometer at 560nm (Erel, 2005).



**Figure 2.** The effect of Ellagic Acid (EA) treatment on serum glucose

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Data are represented as Mean $\pm$ SEM. \*\*\*P<0.001 compared with the control group. Statistical analysis was conducted using one-way ANOVA followed by Tukey's Post-Hoc Test.



**Figure 3.** The effects of Ellagic Acid (EA) treatment on hyperalgesia in the tail-flick test

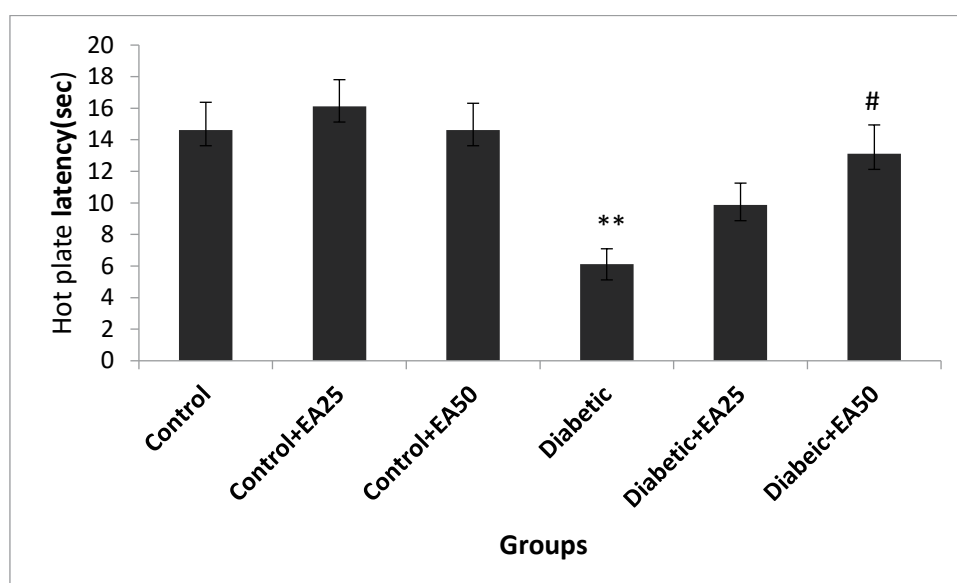
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Data are represented as Mean±SEM. \*P<0.05 compared with the control group; #P<0.05 compared with diabetic rats. Statistical analysis was carried out using one-way ANOVA followed by Tukey's Post-Hoc Test.

The obtained data were expressed as Mean±Standard Error of the Mean (SEM). One-Way Analysis of Variance (ANOVA) followed by Tukey's Post-Hoc Test were used to determine the significant differences between groups. P<0.05 was regarded to be significant.

### 3. Results

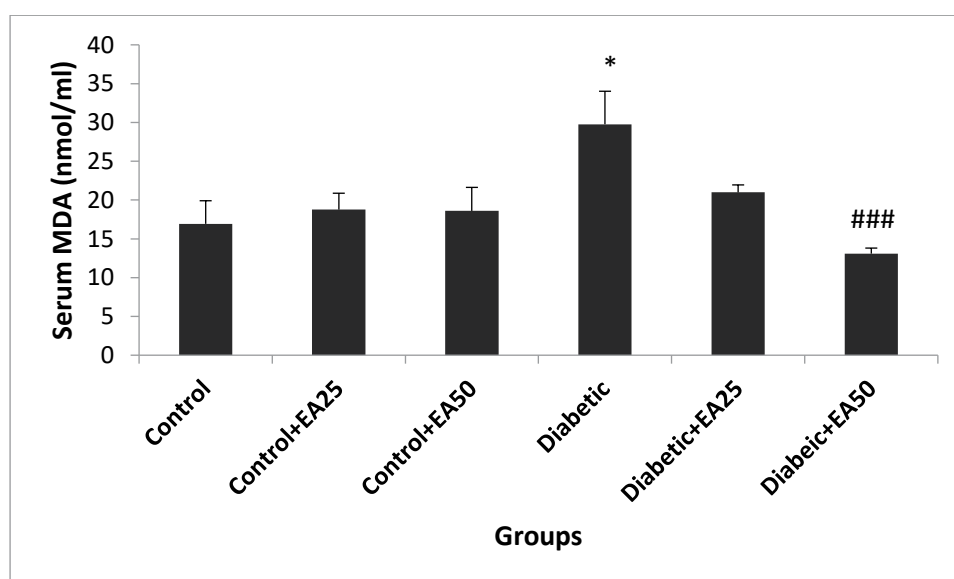
The one-way ANOVA results suggested no significant difference in body weight and plasma glucose level between experimental groups before hyperglycemia induction. Untreated hyperglycemic animals had significantly lower body weight and elevated plasma glucose levels at the end of the study than those of the control group



**Figure 4.** The effects of Ellagic Acid (EA) treatment on hyperalgesia in hot plate test

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Data are represented as Mean±SEM. \*\*P<0.01 compared with the control group; #P<0.05 compared with diabetic rats. Statistical analysis was carried out using one-way ANOVA followed by Tukey's Post-Hoc Test.



**Figure 5.** The effects of Ellagic Acid (EA) treatment on serum Malondialdehyde (MDA)

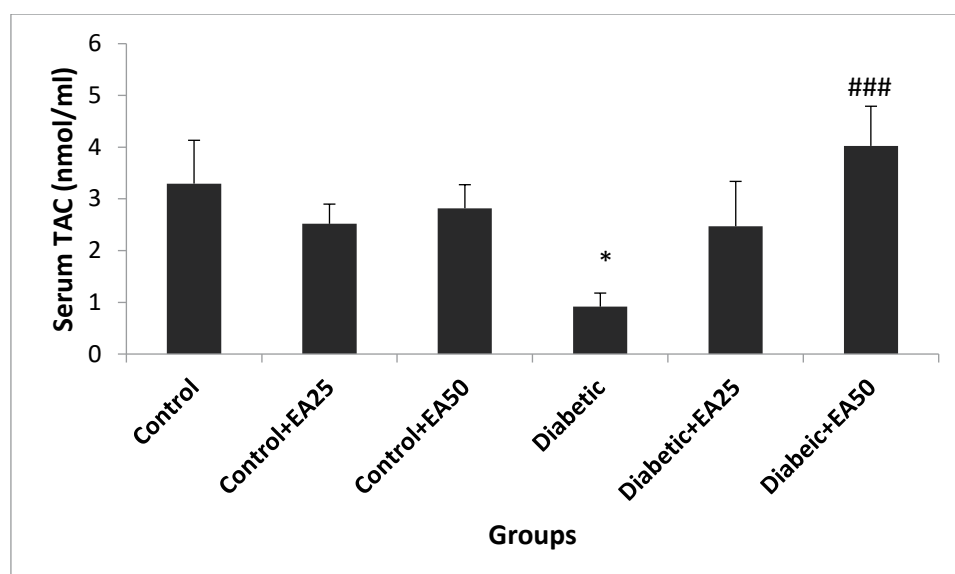
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Data are represented as Mean±SEM. \*P<0.05 compared with the control group; ###P<0.001 compared with diabetic rats. Statistical analysis was performed using one-way ANOVA followed by Tukey's Post-Hoc Test.

(P<0.001). EA treatment at all used doses was ineffective in preventing weight loss and plasma glucose rise in hyperglycemic rats, as did not alter the weight and plasma glucose level in control animals (Figure 1 & 2).

According to the one-way ANOVA results, a significant reduction in the TF latency was observed in untreated hyperglycemic rats, compared with the control group (P<0.05). Hyperglycemic rats treated with EA at a dose

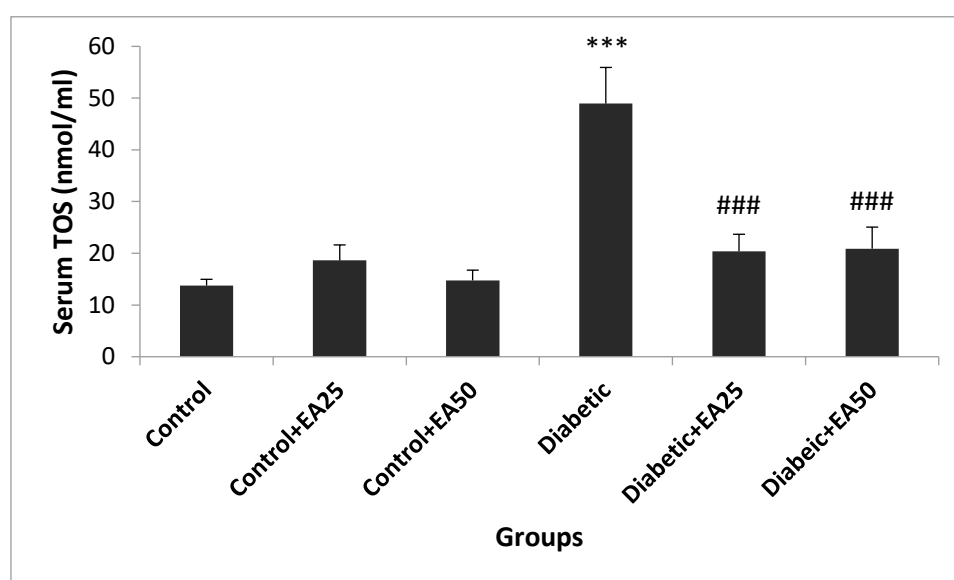
of 25 mg/kg afforded marked longer TF latency than untreated hyperglycemic animals (P<0.05); however, TF latency enhancement in hyperglycemic rats by EA at a dose of 50 mg/Kg was not significant. The administration of both doses of EA caused no significant change in TF latency in control rats compared with the untreated control group (Figure 3).



**Figure 6.** The effects of Ellagic Acid (EA) treatment on serum Total Antioxidant Capacity (TAC)

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Data are represented as Mean±SEM. \*P<0.05 compared with the control group; ###P<0.001 compared with diabetic rats. Statistical analysis was performed using one-way ANOVA followed by Tukey's Post-Hoc Test.



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**Figure 7.** Ellagic Acid (EA) treatment effects on serum Total Oxidant Status (TOS)

Data are represented as Mean $\pm$ SEM. \*\*\*P<0.001 compared with the control group; ###P<0.001 compared with diabetic rats. Statistical analysis was carried out using one-way ANOVA followed by Tukey's Post-Hoc Test.

The one-way ANOVA results revealed a significant decrease in HP latency response in hyperglycemic animals compared with the control group (P<0.01). HP latency deficit in hyperglycemic rats was significantly reversed by EA treatment at a dose of 50 mg/kg (P<0.05). Treatment of the control animals with EA did not alter HP latency response (Figure 4).

The statistical analysis by the one-way ANOVA data confirmed that MDA levels in the serum of the diabetic rats were significantly higher than those of control animals (P<0.05). Moreover, the concentration of TAC in the serum was significantly lower in the hyperglycemic group than in the control group (P<0.05). Additionally, the TOS concentration in the serum of the hyperglycemic group was significantly higher than that of the control group (P<0.001).

EA-treated hyperglycemic groups indicated a significant decrease in the levels of MDA (P<0.001) at a dose of 50 mg/kg and TOS (P<0.001) at both doses (25 and 50 mg/kg), compared with the untreated diabetic group. EA administration to the hyperglycemic animals caused a significant increase in TAC, compared with untreated hyperglycemic animals only at 50 mg/kg (P<0.001).

Treatment with EA t caused no significant difference in oxidative stress markers in control animals compared with untreated controls (Figure 5, 6, 7).

#### 4. Discussion

In the present study, we evaluated the possible therapeutic effect of EA on hypernociception in STZ-induced hyperglycemic rats and also investigated the underlying oxidative stress-related mechanisms.

The obtained results indicated that four weeks of administration of EA at two different doses elicited anti-nociceptive and antioxidant effects in hyperglycemic rats; however, it did not cause an anti-hyperglycemic effect.

STZ, by impelling direct injury in beta cells of pancreatic islets, causes hyperglycemic conditions (Solanki & Bhavsar, 2015). We observed blood glucose enhancement in rats after receiving STZ that is consistent with other studies (Fatani et al., 201).

Several studies reported the development of hypernociception in STZ-induced hyperglycemic animals assessed by different behavioral tests, such as Tail-flick, Tail immersion, Hot-plate, and von Frey filaments (Dobretsov, Hastings, Romanovsky, Stimers, & Zhang, 2003; Kamei, Ohsawa, Miyata, & Tanaka 2008; Kuhad, Bishnoi, Tiwari, & Chopra, 2009). The present study data found a reduction in both TF and HP latencies in STZ-induced hyperglycemic rats, i.e., compatible with other reports (Hajjalizadeh, Nasri, Kaeidi, Sheibani, Rasouljan, & Esmaeili-Mahani, 2014).



The pathophysiology of the DN is indistinct and abstruse; however, hyperglycemia, oxidative stress, inflammation, and apoptosis are possibly involved (Ahmed 2005; Green et al., 2011; Rodrigues et al., 2011; Yang, Jin, Kei Lam, & Yan, 2011). Hyperglycemia induces increased free radicals that affect peripheral and central nervous systems (Coppey, Gellett, Davidson, Dunlap, & Yorek, 2002). The excess formation of ROS causes mitochondrial damage in diabetic neurons under hyperglycemic conditions (Pacher, Obrosova, Mabley, & Szabó, 2005). Furthermore, STZ-induced diabetes is specified by endogenous antioxidant enzymes disturbance (Tolakis & Godin, 1995); thus, the excitation of antioxidant enzymes and a reduction in free radical production may protect against some diabetes complications. The reduced TAC and increased concentrations of MDA and TOS were observed in hyperglycemic animals in the present study. These data were in line with the previous findings that have reported the increased level of MDA and TOS, reduced level of TAC, and decreased Activity of Catalase (CAT) and Superoxide Dismutase (SOD) in STZ-induced diabetic rats (Colak, Geyikoğlu, Aslan, & Deniz, 2014; Mehanna, El Askary, Al-Shehri, & El-Esawy, 2017).

Reports on the direct effective activity of EA on abnormal pain perception in hyperglycemia have not been presented. Therefore, we aimed at testing the ameliorative effect of EA on hyperglycemia-induced hypernociception.

Several antioxidant compounds have been reported to inhibit the induction of DPN significantly. Similarly, in our study, oral administration of EA for four weeks could attenuate the reduction in latencies in TF (at a dose of 25 mg/kg) and HP (at a dose of 50 mg/kg) tests in hyperglycemic rats; however, it did not alter thermal pain threshold of the control animals.

The antioxidant effect of EA is among the primary mechanisms that might cause the effective analgesic property of this substance. The potent antioxidant properties of EA have been demonstrated, such as scavenging free radicals, increasing antioxidant enzyme activity, decreasing LPO, and reducing ROS formation (Chao, Hsu et al., 2009; Uzar et al., 2012; Kiasalari et al., 2017). Accordingly, in the present study, the biochemical analysis of the serum indicated that treatment of hyperglycemic rats with EA decreased the MDA level (at a dose of 50 mg/kg) and TOS (at the doses of 25 & 50 mg/kg) and increased TAC (at the dose of 50 mg/kg). In this regard, EA has been demonstrated to ameliorate learning and memory deficits and mitigate oxidative stress by decreasing MDA and increasing the GSH and CAT activity in a rat model of Alzheimer's disease (Kiasalari et al., 2017). Moreover, EA suppressed oxidative damage of the

sciatic nerve and brain and reduced these tissues' MDA and TOS levels in diabetic rats (Uzar et al., 2012). In our study, EA at the doses and duration used here did not ameliorate STZ-induced hyperglycemia; therefore, the obtained anti-nociceptive effect was not correlated with blood glucose reduction.

An association was indicated between the development of DN and elevated levels of pro-inflammatory cytokines in hyperglycemic rats (Fatani et al., 2015). Previous studies indicated that EA exerts protective effects against inflammation and apoptosis. Rizk reported that EA treatment relieved the neuronal injury induced by toxic agents via ameliorating pro-inflammatory cytokines, attenuating oxidative stress markers, and exerting antiapoptotic properties (Rizk, Masoud, & Maher, 2017). Similarly, it was suggested that treatment with EA protects rats from ischemic brain injury via inhibiting inflammatory reactions and apoptosis (Chen, Zheng, & Wang, 2016). Therefore, such anti-inflammatory and anti-apoptotic mechanisms may have occurred in our study by EA to protect against DN.

Our study investigated some antioxidant mechanisms involved in the anti-nociceptive activities of EA in hyperglycemic rats. Moreover, other studies have explained the analgesic action of EA by mediation through opioidergic and NO-cGMP pathways and inhibition of cyclooxygenase activity (Naghizadeh, Mansouri, & Ghorbanzadeh, 2016). However, additional studies are required to clarify the mechanisms underlying the anti-nociceptive activity of EA.

In the present study, EA did not alter pain perception and oxidative stress markers, including MDA, TOS, and TAC in control rats, probably to maintain their physiological balance. This finding can be confirmed because several antioxidant compounds that had no effects on pain threshold and oxidative stress criteria in intact rats indicated considerable effects on these parameters in STZ- hyperglycemic rats (Prince & Kamalakkannan 2006, Mirshekar, Roghani, Khalili, Baluchnejadmojarad, & Arab Moazzen, 2010). Elevated ROS production plays a crucial role in the pathogenesis of diabetes complications, such as hyperalgesia; thus, EA could improve the oxidative status and pain perception in hyperglycemic conditions by its antioxidant properties. There was no imbalance in oxidative status; therefore, anti-oxidant properties of EA could not alter these parameters.

Finally, a limitation of this investigation is the induction of diabetes type I through STZ injection. While it could be more appropriate to use transgenic rats, it was impossible for us. Furthermore, we studied only the antioxidant



mechanism, while the evaluation of additional mechanisms can fortify the utility of EA to ameliorate hyperglycemic hypernociception.

## 5. Conclusion

Overall, our findings indicated that STZ-induced hyperglycemia causes increased nociception and oxidative stress. EA could mitigate hyperalgesic state and suppress oxidative stress in hyperglycemic rats. Therefore, the antioxidant properties of EA may be involved in this protective effect; however, further studies are required to explain other underlying mechanisms. Our results suggested that EA could be a promising agent for managing abnormal pain perception associated with diabetes.

## Ethical Considerations

### Compliance with ethical guidelines

The animal care and treatment procedures were approved by the Ethics Committee of the Hamadan University of Medical Sciences (Code: IR.UMSHA.REC.1395.284).

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### Authors' contributions

All authors equally contributed to preparing this article.

### Conflict of interest

The authors declared no conflicts of interest.

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