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Gallic Acid Mitigates Carrageenan-Induced Acute Paw Edema in Rats through Antioxidant Activity and Modulation of NF-κB, COX-2, and Proinflammatory Cytokines

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ABSTRACT

This research project was conducted to assess the anti-inflammatory and antioxidant mechanisms of gallic acid (GA) in a rat model of paw edema triggered by carrageenan (Carr). In this experimental investigation, 42 adults male Wistar rats were casually sorted to make six experimental groups. Group I received normal saline (NS). Group II received NS before the Carr injection. Group III was treated with indomethacin (IND) prior to Carr injection. Groups IV-VI received GA at increasing doses before the Carr administration. By analyzing oxidative stress indicators, inflammatory signaling pathways, and serum cytokine levels, the regulating properties of GA on Carr-stimulated rat paw inflammation were ascertained. The levels of cyclooxygenase-2 (COX-2), prostaglandin E2 (PGE2), nuclear factor-kappa B (NF-κB), malondialdehyde, nitric oxide, and tumor necrosis factor α (TNF-α) were all meaningfully reduced by GA. Additionally, GA increased interleukin-10 secretions and enhanced the activation of glutathione peroxidase, catalase, and superoxide dismutase besides glutathione content. Gallic acid meaningfully reduced paw edema triggered via Carr injection in rats, indicating a robust acute anti-inflammatory effect. Moreover, it suppressed Carr-induced inflammation through its antioxidative properties.

Keywords: Gallic acid, Carrageenan, Antioxidant, NF-κB, TNF-α

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INTRODUCTION

Inflammation serves as both a healing mechanism to repair damaged tissues and as a protective response to eliminate harmful stimuli [1]. Traditionally, it is identified by five key signs: swelling, heat, redness, pain, and impaired tissue function, which reflect increased vascular endothelial porousness and the migration of immune cells into the crisis zone [2]. Several anti-inflammatory medications are used to reduce the impairments caused by inflammatory mediators. These medications, besides their effectiveness against inflammation, have several side effects [3]. Therefore, finding a new therapeutic strategy against inflammatory consequences was a research interest for scientists.

Gallic acid (GA, a trihydroxybenzoic acid) is a phytochemical known for its diverse pharmacological characteristics, particularly its antioxidant and anti-inflammatory effects [4]. GA can be extracted from different fruits such as berries, grapes, mango, areca nut, walnut, oak bark, green tea, and red wine [5]. Studies have confirmed that GA decreases the generation of pro-inflammatory factors, for instance interleukin-18 (IL-18), IL-1β, IL-6 and tumor necrosis factor-α (TNF-α) via downregulation of nuclear factor-κB (NF-κB). Moreover, it downregulates the expression intensity of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) [6-9]. Additionally, GA shows antioxidant properties by improving the performance of key cellular enzymatic antioxidants, in particular glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) [4, 10].

Experimental paw edema triggered by carrageenan (Carr), a recognized acute inflammation model, is commonly employed for evaluating new anti-inflammatory constituents and induces a biphasic edema in the sub-plantar surface of rat paws. Following carrageenan injection, the early step of edema is initiated by the release of serotonin, bradykinin, and histamine and the generation of prostaglandins by COX enzymes. The delayed phase begins after neutrophil permeation and the subsequent continuation of prostaglandin synthesis [11]. Paw edema stimulated by Carr is extremely susceptible to nonsteroidal anti-inflammatory drugs (NSAIDs), making it a useful test for finding novel anti-inflammatory agents [12]. Given the significant etiological role that inflammation and oxidative damage play in paw edema stimulated by carrageenan, alongside the anti-inflammatory and

antioxidant characteristics of GA, it was theorized that GA could serve as a plausible substance to alleviate acute inflammation stimulated by carrageenan. Accordingly, this project seeks to ascertain the underlying biological processes responsible for the anti-inflammatory properties of GA in Carr-triggered paw edema in rats.

MATERIALS AND METHODS

Chemicals

λ-Carr and GA were attained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Indomethacin (IND) was prepared from Iran-Daru Pharmaceutical Company in Tehran, Iran. Every other chemical was from the Merck Company in Darmstadt, Germany, and was of analytical quality.

Animals

Forty-two adult male rats (Wistar, 200-220 g) were included in this experiment. The animals were retained in a light/dark pattern (12:12), and the environmental control system was set to preserve the steady temperature at 22 ± 2°C and the approximate humidity at 55–60%. They had unrestricted accessibility to a usual rodent pellet diet and drinking water. All handling and experimental arrangements were supervised, adhering to the strategies of the Local Ethics Committee for the Control and Supervision of Laboratory Animal Experiments under the authority of the Mazandaran University of Medical Sciences, Sari, Iran, and institutional legislation (IR.MAZUMS.REC.1398.447).

Experimental Design

The rats (n=7/group) were casually sorted to make six experimental groups. Group I was given normal saline (NS) intraperitoneal (5 mL/kg, i.p.) thirty min before injecting NS (100 µL, intraplantar (i.pl.)) into the rat's right paw. Group II was given NS (5 mL/kg, i.p.) thirty min before injecting Carr 1% (100 µL, i.pl.) into the rat's right paw. Group III received IND (5 mg/kg, i.p.) thirty min before injecting Carr 1% (100 µL, i.pl.) into the rat's right paw. Groups IV-VI received GA (at doses of 100, 200, and 400 mg/kg, respectively, i.p.) thirty min before injecting Carr 1% (100 µL, i.pl.) into the right paw of rats.

Acute Paw Edema Triggered via Carr Injection

Paw inflammation in the rats was triggered by the administration of Carr in the intraplantar section of the right hind paw. A plethysmometer (Ugo Basile Co., Italy) was applied to appraise inflammation in the paw immediately before and at 0.5, 1, 2, 3, 4, and 5 h after the Carr injection [13]. The extent of inflammation was determined by applying the following equation [14]:

% Inflammation = $((V2 - V1) / V1) * 100$

The right hind paw's volume (mL) beforehand and five hours following the Carr injection was indicated by V1 and V2, respectively.

Sample Collection

After evaluation of paw edema, a mixture of xylazine hydrochloride (3 mg/kg, i.p.) and ketamine hydrochloride (50 mg/kg, i.p.) was used to deeply anesthetize the rats, then they were euthanized, and their right hind paws were cut off. Hind paw pad tissue samples were taken from rats. For each sample, the pieces were blended in a cold buffer dilution (0.1 M Tris-HCl, 1/10 w/v) using a homogenizer, and the clarified supernatants were collected by centrifuging the mixture at 12,000 g for fifteen min at 4°C for oxidant and inflammatory marker measurements. The concentration of protein in the liquid sample was individually analyzed utilizing Bradford's procedure.

Measurement of Malondialdehyde

Malondialdehyde (MDA) contents were quantified by the thiobarbituric acid (TBA) performance declared by Ohkawa *et al*. [15]. This procedure is based upon the reaction with MDA and TBA, and it leads to a colorful mixture. The absorbance at 532 nm was determined to quantify MDA concentration in the complex. The results were clarified as nmol/gr of tissue.

Measurement of Nitric Oxide

The levels of nitric oxide (NO) were measured by the Griess reaction as stated by Wang *et al*. [16]. In short, a portion of the supernatant was added to the Griess reagent to form a colored compound. Absorbance of this compound was quantified at 540 nm, and NO concentration was realized, conforming to the standard curve.

Measurement of Reduced Glutathione

Reduced glutathione (GSH) contents were quantified adhering to the assay declared by Beutler *et al*. [17]. In summary, 10% trichloroacetic acid was used to deproteinize tissue homogenates, and the tissues were centrifuged at 3500 rpm for 10 min. A 50 µL of clarified supernatant was blended with a (disodium hydrogen phosphate (0.32 mol/L), 5,5′-dithiobis-2-nitrobenzoic acid (0.04%, DTNB)) mixture. Aliquots of the reaction were then spectrophotometrically quantified at 412 nm for the yellow-colored compound generated. Values were calculated as GSH contents in mmol per gram of tissue.

Measurement of SOD Activity

The procedure described by Masayasu and Hiroshi was applied to determine the activity of SOD in tissue homogenates [18]. The process is based on the autoxidation of pyrogallol to form superoxide anions ($O_2^{\bullet-}$). Subsequently, nitroblue tetrazolium (NBT) is employed to detect the consequent $O_2^{\bullet-}$, yielding formazan that can be quantified. The development of formazan color intensity is directly proportional to the amount of $O_2^{\bullet-}$ that is scavenged by SOD. Its activity was determined and stated as units/gram of tissue.

CAT Activity Assay

The activity of CAT was assessed adhering to Osumi and Hashimoto [19]. They monitored decomposition of the hydrogen peroxide by tracking the decline in the absorbance at 240 nm. The readings were determined at a period of 3 min at 25°C, and its activity was calculated in units/g tissue.

GPx Activity Assay

The activity of GPx was quantified with the help of a Ransel kit (Randox Laboratories Ltd, Crumlin, UK). In this procedure, the proportion of NADPH oxidation is quantified by the decline in the absorbance at 340 nm. A single unit of its activity is characterized as the volume of enzyme mandatory to oxidize 1 nmol of NADPH/min [20].

ELISA Measurements in Paw Tissue

Levels of TNF-α, IL-10, and prostaglandin E2 (PGE2) were appraised, conforming to the manufacturer's directions with enzyme immunoassay kits (MyBioSource; San Diego, USA). The color absorbance obtained was quantified at 450 nm (OD450) with the help of a multiwell plate reader. The limits of quantitation for TNF-α and IL-10 were 15.6 pg/mL and 31.2 pg/mL, respectively, and the sensitivity for PGE2 was 0.1 ng/mL. Moreover, the levels

of cyclooxygenase-2 (COX-2) were measured through an ELISA kit from IBL International GmbH (Germany) with a detection range of 0.36–23 ng/mL. Moreover, NF-κB p65 extent in paw tissue was assessed by an ELISA kit from Elabscience (China) (adhering to the provider's guidelines). The threshold of this ELISA kit was calculated as 46.88 pg/mL.

Statistical Analysis

All values were provided as mean ± standard deviation. Statistical assessments were conducted using one-way ANOVA, followed by Tukey's post-hoc test. Significance was set at $P<0.05$. Software called GraphPad Prism 6 (GraphPad Software Inc., San Diego, USA) was allocated for all statistical procedures.

RESULTS

Effects of GA on Inflammation

Contrasted to the Carr group, the indomethacin (IND) group's data disclosed a noteworthy decrease in right hind paw inflammation (Fig. 1b, $p<0.05$). Gallic acid (GA) pretreatment at 100, 200, and 400 mg/kg dosages, administered thirty min prior to Carr injection, significantly diminished right hind paw inflammation compared to the Carr group (Fig. 1b, $p<0.05$). Additionally, our study found that IND reduced right hind paw inflammation at all time points evaluated versus the Carr group (Fig. 1a, $p<0.05$). Preliminary management with GA at 100 mg/kg resulted in the reduction of inflammation in the right hind paw 4 hours following Carr injection, versus the Carr group ($p<0.05$). In comparison to the Carr group, pretreatment with GA at 200 and 400 mg/kg meaningfully diminished inflammation in the right hind paw 1 hour after Carr administration (Fig. 1a, $p<0.05$).

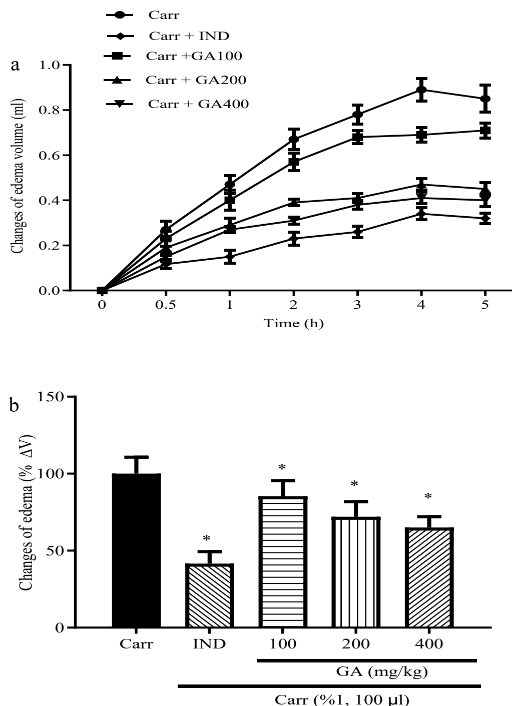

Fig. 1 Effect of GA and IND against Carr-induced paw edema. The treatments were delivered 30 min prior to Carr injection. (*) represents a statistically noteworthy difference from the Carr-treated group ($p<0.05$). Data are shown as mean ± SD, and statistical analysis was accomplished using one-way ANOVA followed by Tukey's post-hoc test (n=7); GA: Gallic acid, IND: Indomethacin.

Effects of GA on COX-2 and PGE2 Levels

As illustrated in Fig. 2a-b, the Carr group had considerably higher levels of PGE2 and COX-2 than the NS group ($p<0.05$). Paw tissues' levels of COX-2 were meaningfully diminished by GA preliminary treatment at amounts of 200 and 400 mg/kg versus the Carr group (Fig. 2a, $p<0.05$). Contrasted to the Carr group, GA at all doses meaningfully reduced the PGE2 level (Fig. 2b, $p<0.05$).

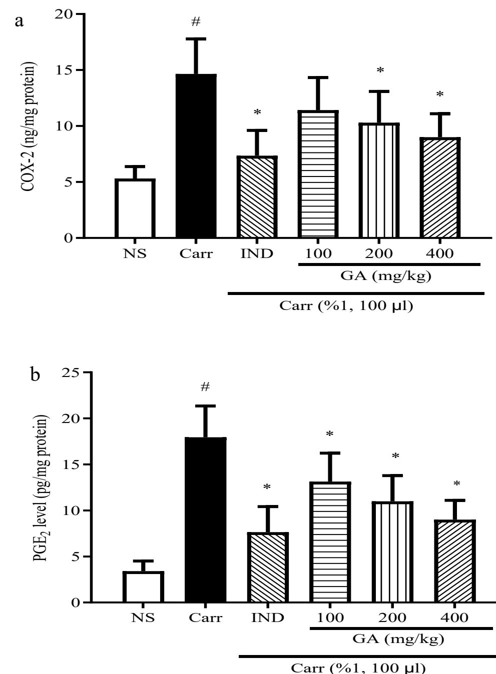

Fig. 2 Effect of GA and IND on COX-2 (a) and PGE2 (b) levels of paw tissue. The treatments were delivered 30 min prior to Carr injection. (#) represents a statistically noteworthy difference from the normal NS group ($p<0.05$). (*) represents a statistically noteworthy difference from the Carr-treated group ($p<0.05$). Data are shown as mean ± SD, and statistical analysis was accomplished using one-way ANOVA followed by Tukey's post-hoc test (n=7); COX-2: Cyclooxygenase-2, PGE2: Prostaglandin E2.

Effects of GA on IL-10, NF-κB, and TNF-α Levels

In comparison to the NS group, the concentrations of NF-κB and TNF-α in the Carr group were considerably higher (Fig. 3b-c, $p<0.05$). The amount of NF-κB was considerably lowered by GA at all doses compared to the Carr group (Fig. 3c, $p<0.05$). Contrasted to the NS group, the amount of the anti-inflammatory cytokine IL-10 was meaningfully inferior in the Carr group. However, preliminary treatment with GA at a dose of 400 mg/kg meaningfully promoted the amount of IL-10 in paw tissues (Fig. 3a, $p<0.05$).

Effects of GA on GPx Activity and GSH Content

As seen in Fig. 4, contrasted to the NS group, the Carr group's GPx activity and GSH content were considerably lower ($p<0.05$). The GSH level in paw tissues was meaningfully greater after GA injection at a dose of 400 mg/kg in opposition to the Carr group (Fig. 4a, $p<0.05$). Besides, in contrast to the Carr group, pretreatment with GA at 200 and 400 mg/kg markedly boosted the GPx activity in paw tissues (Fig. 4b, $p<0.05$). Besides, administering IND thirty min prior to i.pl. Carr injection markedly prompted GPx activity and GSH content in opposition to the Carr group ($p<0.05$), as illustrated in Fig. 4a-b.

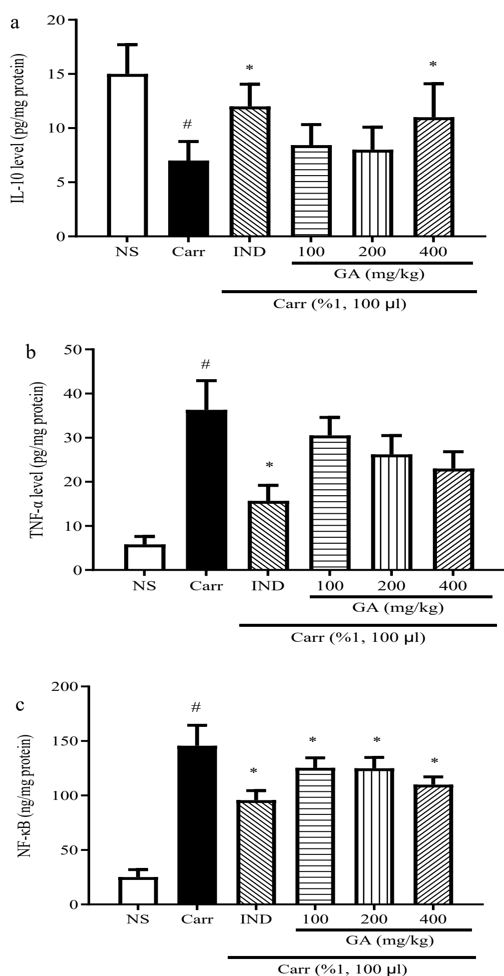

Fig. 3 Effect of GA and IND on IL-10 (a), TNF-α (b), and NF-κB (c) levels of paw tissue. The treatments were delivered 30 min prior to Carr injection. (#) represents a statistically noteworthy difference from the normal NS group ($p<0.05$). (*) represents a statistically noteworthy difference from the Carr-treated group ($p<0.05$). Data are shown as mean ± SD, and statistical analysis was accomplished using one-way ANOVA followed by Tukey's post-hoc test (n=7). IL-10: Interleukin-10, TNF-α: Tumor necrosis factor-α, NF-κB: nuclear factor-κB, NS: normal saline.

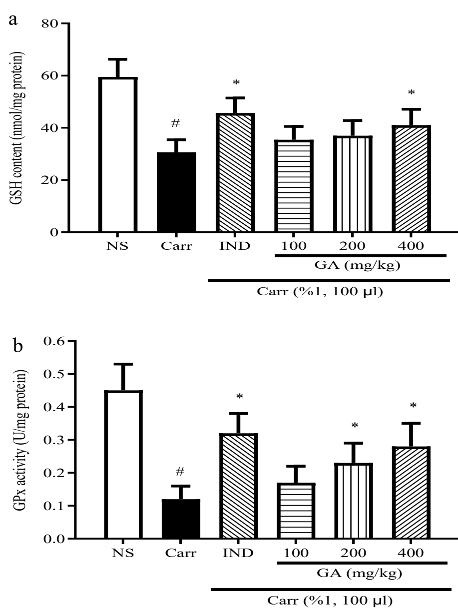

Fig. 4 Effect of GA and IND on GSH content (a) and GPx activity (b) of paw tissue. The treatments were delivered 30 min prior to Carr injection. (#) represents a statistically noteworthy difference from the normal NS group ($p<0.05$). (*) represents a statistically noteworthy difference from the Carr-treated group ($p<0.05$). Data are shown as mean ± SD, and statistical analysis was accomplished using one-way ANOVA followed by Tukey's post-hoc test (n=7). GSH: Reduced glutathione, GPx: glutathione peroxidase.

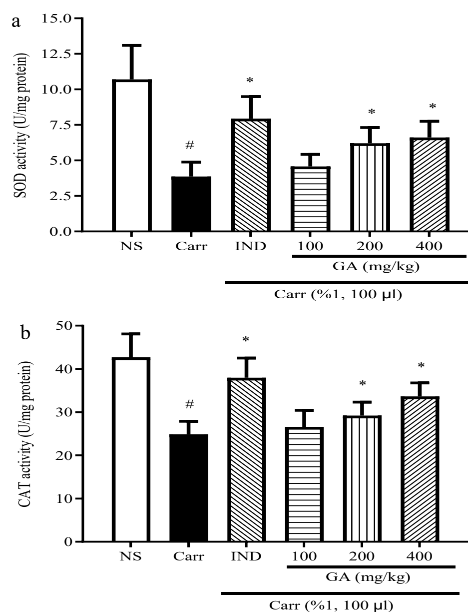

Fig. 5 Effect of GA and IND on SOD (a) and CAT (b) activities of paw tissue. The treatments were delivered 30 min prior to Carr injection. (#) represents a statistically noteworthy difference from the normal NS group ($p<0.05$). (*) represents a statistically noteworthy difference from the Carr-treated group ($p<0.05$). Data are shown as mean ± SD, and statistical analysis was accomplished using one-way ANOVA followed by Tukey's post-hoc test (n=7). SOD: Superoxide dismutase, CAT: Catalase.

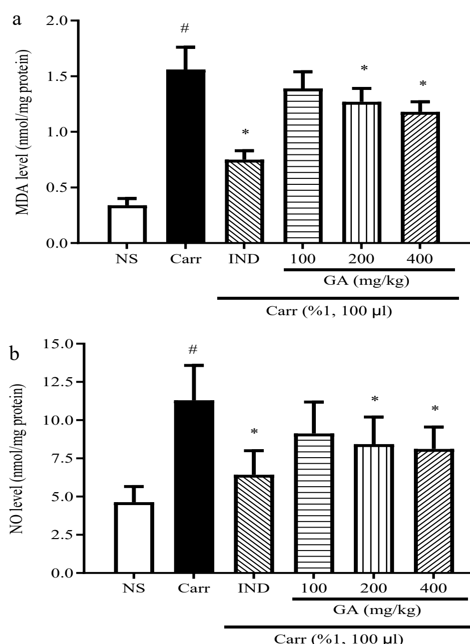

Fig. 6 Effect of GA and IND on MDA (a) and NO (b) levels of paw tissue. The treatments were delivered 30 min prior to Carr injection. (#) represents a statistically noteworthy difference from the normal NS group ($p<0.05$). (*) represents a statistically noteworthy difference from the Carr-treated group ($p<0.05$). Data are shown as mean ± SD, and statistical analysis was accomplished using one-way ANOVA followed by Tukey's post-hoc test (n=7). MDA: Malondialdehyde, NO: Nitric oxide.

Effects of GA on SOD and CAT Activities

As illustrated in Fig. 5a-b, the Carr group had significantly lower SOD and CAT activity contrasted to the NS group ($p<0.05$). Pretreatment with GA at 400 mg/kg meaningfully improved the activity of CAT in paw tissues contrasted to the Carr group (Fig. 5b, $p<0.05$). Additionally, preliminary management with GA at 200 and 400 mg/kg markedly boosted the activity of SOD in paw tissues versus the Carr group (Fig. 5a, $p<0.05$). Moreover, the injection of IND thirty min prior to i.pl. Carr administration meaningfully improved CAT and SOD activity in comparison with the Carr group (Fig. 5a-b, $p<0.05$).

Effects of GA on NO and MDA levels

Comparing the Carr group to the NS group, the NO and MDA contents were meaningfully higher in the Carr group (Fig. 6a-b, $p<0.05$). Furthermore, paw tissues' MDA and NO levels were considerably lower after pretreatment with GA at 200 and 400 mg/kg contrasted to the Carr group (Fig. 6a-b, $p<0.05$). Similarly, contrasted to the Carr group, administering IND thirty minutes before the i.pl. injection of Carr dramatically reduced MDA and NO (Fig. 6a-b, $p<0.05$).

DISCUSSION

As a pro-inflammatory cytokine, tumor necrosis factor-alpha regulates various inflammatory mediators, including NO, PGE2, COX-2, etc. [21-23]. The present research study found that following carrageenan injection to induce paw edema, there was an excess in the formation of the pro-inflammatory intermediaries COX-2 and TNF-α. However, treatment with GA meaningfully diminished the elevated expression levels of these cytokines. Additionally, the administration of indomethacin (IND), a standard anti-inflammatory drug, thirty min prior to the i.pl. Carr injection evidently decreased serum concentrations of COX-2 and TNF-α. Because of its cyclooxygenase (COX)-dependent mechanism, the arachidonate cyclooxygenase inhibitors effectively regulate the Carr-stimulated paw swelling. Based on the acquired data, it is hypothesized that gallic acid (GA) could have arachidonate COX suppressive characteristics. These findings support the previous observation reporting that gallic acid suppresses hepatotoxicity by attenuating the secretion of inflammatory factors, in particular COX-2 and TNF-α [24].

According to reports, flavonoids impede the cyclooxygenase cascades involved in arachidonate metabolism [25]. Therefore, the reduction in TNF-α concentration, along with decreased concentrations of COX-2 and PGE2 in the paw, appears to be a contributing factor to the anti-inflammatory influences of GA. In conformity with our outcomes, Zhou *et al*. indicated that gallic acid-selenium nanoparticles could meaningfully lower the concentration of inflammatory mediators, including TNF-α, in an in vivo evaluation of acute renal failure [26]. Additionally, the results obtained from the research work of Li and colleagues demonstrated that GA attenuated inflammatory mediators like TNF-α in the cornea, conjunctiva, and macrophage RAW264.7 [27].

The expression of NF-κB is definitely linked to pro-inflammatory factors, in particular IL-6 and TNF-α [28]. Hydroxyl radicals (from the NO-peroxide reaction) or peroxynitrite (a product of the NO-superoxide reaction) may induce lipid peroxidation and arachidonic acid release from the cellular membrane, thereby stimulating COX-2 [29]. Therefore, detecting COX-2 inhibitors has been thought of as a promising manner to reduce inflammation. Our findings specified that GA had a suppressive impact on the mobilization of transcription factor NF-κB and the formation of inflammatory factors TNF-α and NO by carrageenan in a paw edema model. Furthermore, our research found that GA significantly reduced COX-2 in Carr-induced mice paw edema and decreased PGE2 synthesis in a dose-related manner. These findings implied that GA exerted its anti-inflammatory performance partly by the prevention of COX-2 protein expression and subsequent diminution of the proinflammatory mediator, PGE2. GA has been illustrated to impede the generation of inflammatory markers, for instance TNF-α, COX-2, and NO, by hampering NF-κB activity [27]. Furthermore, Morais *et al*. have revealed that GA could prevent TNF-α-stimulated NF-κB activation. It has been speculated that free radicals are essentially involved in the Carr-induced acute inflammatory response [30].

Interleukin-10 (IL-10) is a major anti-inflammatory mediator involved in regulating immune responses. It prevents the formation of TNF-α, IL-1, IL-6, IL-8, and IL-12 by monocytes and macrophages and has a premier role in the management of the immunological responses [31]. Our results also indicated that carrageenan could diminish the expression of IL-10 levels in the paw. For the first time, it has been demonstrated that GA meaningfully improved paw IL-10 levels following an acute inflammatory challenge. According to an in vitro investigation, GA successfully raised IL-10 hepatocellular carcinoma cell levels [32]. Furthermore, Zhu *et al*. reported that pretreatment with GA notably restored IL-10 expression, which had been reduced in vitro and in vivo in ulcerative colitis [33].

Investigations have found that Carr-induced inflammation is closely associated with a rise in oxidative stress [34, 35]. Our results indicated that pretreatment with high doses of GA significantly enhanced GPx, CAT, and SOD activities and GSH concentration in Carr-induced rats. Additionally, MDA levels, which reflect a byproduct of lipid peroxidation, were markedly reduced in the medium- and high-dose GA + Carr groups. According to recent studies, GA has been shown to reduce the detrimental effects of paraquat by affecting the levels of MDA, GSH, SOD, CAT, and GPx in male rats with paraquat-induced kidney damage [36]. Moreover, GA has been shown to eliminate the disturbances of MDA, GSH, SOD, and CAT levels due to fluoxetine-triggered oxidative stress and hepatic dysfunction in rats [37].

CONCLUSION

According to our findings, GA has a promising anti-inflammatory property against Carr-stimulated acute localized inflammation in rats, like indomethacin. GA may exert preservative properties against oxidative stress by amplifying GSH levels, activating SOD, CAT, and GPx, and diminishing NO and MDA concentrations. This ability to reduce oxidative stress, coupled with an improved IL-10 synthesis and blockage of NF-κB, COX-2, PGE, and TNF-α generation, underlies its anti-inflammatory actions. More research is required to fully clarify how NF-κB function mediates the guardian effects of GA in acute inflammation.

Conflict of Interest

The authors declare that they have no competing interests.

Authors' Contributions

Gholamreza Houshmand conceptualized the project, supervised the work, and administered the project. Mitra Mahmoudi contributed to conceptualization and methodology. Sara Bayat handled visualization and contributed to the investigation. Kobra

Bahrampour Juybari wrote, reviewed, and edited the manuscript and worked on methodology. Pouya Noorian conducted the formal analysis and validated the findings. Foroogh Rostami Ghadi prepared the original draft and contributed to the methodology. Mehdi Goudarzi curated the data and contributed to the methodology.

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Ethical Considerations

Animal handling and experimental procedures were conducted under the oversight of the Local Ethics Committee for the Control and Supervision of Laboratory Animal Experiments under the authority of the Mazandaran University of Medical Sciences, Sari, Iran, and institutional legislation (IR.MAZUMS.REC.1398.447).

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