

Original Article

Protective Effect of Chlorogenic Acid on Methyl Methanesulfonate-Induced DNA Damage in Mouse Spleen Tissue

Mohammad Hossein Pazandeh^{1,2}, Ali Mohammad Ahadi³, Monir Doudi⁴, Amir Nili-Ahmadabadi^{1,2*}

¹ Medicinal Plants and Natural Products Research Center, Hamadan University of Medical Sciences, Hamadan, Iran.

² Department of Pharmacology and Toxicology, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, Iran.

³ Department of Genetics, College of Science, University of Shahrekord, Shahrekord, Iran.

⁴ Department of Microbiology, Fal. C Islamic Azad University, Isfahan, Iran.

*Corresponding author:

Prof. Amir Nili-Ahmadabadi, Department of Pharmacology and Toxicology, School of Pharmacy, Hamadan University of Medical Sciences, Hamadan, Iran. Tel: +98-8138381591, Postal code: 6517838678, Fax: +98-8138380031. Email: a.niliahmadabadi@umsha.ac.ir

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Abstract

Background and Objective: Chlorogenic acid (CGA) is a prominent bioactive compound with diverse pharmacological properties. This study aimed to evaluate the potential protective effects of CGA against methyl methanesulfonate (MMS)-induced genotoxicity in the spleen of male BALB/c mice.

Methods: A total of 36 male BALB/c mice were randomly allocated to six experimental groups (n=6 per group). The control group received normal saline (NS; 1 mL/kg) daily for seven consecutive days. The second group received NS (1 mL/kg) for seven days, followed by a single intraperitoneal (i.p.) injection of MMS (10 mg/kg) one hour after the last NS administration. The third group was treated with CGA (40 mg/kg/day) alone for seven days. The fourth, fifth, and sixth groups were pretreated with CGA at doses of 10, 20, and 40 mg/kg/day, respectively, for seven days; on day 7, one hour after the final CGA dose, each of these groups received a single i.p. injection of MMS (10 mg/kg). Approximately 3 h after MMS injection, all mice were humanely euthanized (ketamine/xylazine), and spleen tissues were collected for subsequent histopathological and biochemical analyses.

Results: MMS treatment significantly increased DNA damage in spleen cells, as indicated by the comet assay, and this genotoxic effect was accompanied by elevated caspase-3/7 activities. Pretreatment with CGA attenuated MMS-induced DNA damage, with reduced DNA migration observed at 10 mg/kg ($P<0.05$) and 20 mg/kg ($P<0.001$) CGA doses. CGA also markedly decreased caspase-3/7 activity in spleen tissue at all tested doses. These biochemical findings were supported by histopathological analysis, which showed preservation of normal splenic architecture in the CGA-pretreated groups against MMS-induced damage.

Conclusion: This study demonstrates that CGA protects against MMS-induced genotoxicity in murine spleen tissue. CGA pre-treatment significantly reduced DNA damage and suppressed caspase-3/7 activity, indicating an inhibitory effect on MMS-induced apoptosis. These findings highlight the chemoprotective potential of CGA against genotoxic chemicals.

Keywords: Chlorogenic acid, Comet assay, Genotoxicity, Methyl methanesulfonate

Introduction

Coffee is among the most widely consumed beverages, with growing scientific interest in its bioactive phytochemicals [1]. Chlorogenic acid (CGA), the predominant phenolic in unroasted coffee beans, often acts in concert with related compounds such as caffeic acid to combat oxidative stress by scavenging reactive oxygen species (ROS) and protecting intracellular macromolecules [2]. Epidemiological evidence links coffee intake with reduced cancer risk, implicating coffee's influence on cell growth regulation, apoptosis, and DNA repair, and highlighting CGA as a promising candidate in natural therapeutics [3].

In some Asian and African countries, approximately 80% of the population relies on traditional medicine for accessibility and affordability [4]. Medicinal plants rich in alkaloids, flavonoids, and phenolics, such as CGA, represent a vast resource for cancer prevention [5]. However, the complexity of plant matrices presents challenges for assessing their safety, efficacy, and mechanisms of action, underscoring the need for rigorous phytochemical and toxicological evaluations to inform their integration into modern healthcare.

A critical area of research is the protection against genotoxic agents. Methyl methanesulfonate (MMS) is a potent alkylating agent that induces DNA damage via ROS generation and alkylation, leading to strand breaks and cell death [6]. Agents that inhibit alkylation and scavenge radicals may mitigate MMS-induced genotoxicity. Consequently, plant-derived compounds such as CGA are of growing interest as potential protective agents against chemically induced DNA damage and mutagenesis [7].

The spleen, a secondary lymphoid organ, orchestrates critical immune surveillance and hematopoiesis, rendering it highly susceptible to circulating genotoxic agents. Its unique architecture houses rapidly proliferating lymphocyte populations, making it a prime target for MMS-induced DNA alkylation and strand breaks. Furthermore, the organ's role in filtering blood and recycling senescent erythrocytes exposes it to systemic damage. Investigating genoprotection in these tissues provides a physiologically relevant window into the systemic immunotoxicity of alkylating

agents [8, 9]. Therefore, evaluating the antigenotoxic potential of chlorogenic acid in the spleen offers crucial insights into safeguarding systemic immune integrity from environmental mutagens.

This study aimed to evaluate the protective effects of CGA against MMS-induced genotoxicity in mouse spleen tissue. These findings aim to elucidate the chemopreventive potential of CGA and contribute to the evidence base for natural agents in genotoxicity mitigation.

Methods

All chemicals were purchased from Merck (Darmstadt, Germany) unless otherwise stated. Chlorogenic acid and methyl methanesulfonate were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Xylazine and ketamine hydrochloride were purchased from Sinared Kala Co. and Exir Pharmaceutical Co..

Animals

Male BALB/c mice (25–30 g) were obtained from the animal house of the Royan Institute (Tehran, Iran). Animals were kept under controlled conditions (25°C, 50–55% relative humidity, 12 h light/dark cycle) at the animal house of Islamic Azad University, Falavarjan Branch. The experimental protocol was approved by the Animal Ethics Committee of Hamadan University of Medical Sciences (IR.UMSHA.AEC.1404.021).

Experimental Protocol and Groups

A total of 36 male BALB/c mice were randomly allocated to six experimental groups (n = 6 per group). The control group received normal saline (NS, 1 mL/kg) daily for seven consecutive days. The second group received NS (1 mL/kg) for seven days, followed by a single intraperitoneal (i.p.) injection of MMS (10 mg/kg) one hour after the last NS administration. The third group was treated with CGA (40 mg/kg/day) alone for seven days. The fourth, fifth, and sixth groups were pretreated with CGA at doses of 10, 20, and 40 mg/kg/day, respectively, for seven days; on day 7, one hour after the final CGA dose, each of these groups received a single i.p. injection of MMS (10 mg/kg). Approximately 3 h after the injection of MMS, all mice were humanely euthanized

(Ketamine/Xylazine), and spleen tissue was collected for subsequent histopathological and biochemical analyses [7].

Comet Assay

The comet assay is a sensitive and versatile technique that can be used to evaluate the genotoxic potential of chemicals, including DNA damage caused by strand breaks. Briefly, tissue-derived cell suspensions were mixed with 200 μ L of 0.6% low-melting-point agarose and added to pre-coated microscope slides treated with 1% normal-melting-point agarose gel. A coverslip was added on top of the slides to help facilitate even distribution of the tissue and cell mixture across slides; after gel solidification, the coverslip was gently lifted off, and the microscope slides were placed in lysis solution (2.5 M sodium chloride, 100 mM ethylenediaminetetraacetic acid [EDTA], 10 mM tris(hydroxymethyl)aminomethane hydrochloride [Tris-HCl], 1% Triton X-100, and 10% dimethyl sulfoxide) for one hour at 4°C. After lysis, the slides were rinsed with distilled water for 30 minutes. The electrophoresis working solution was prepared at 4°C (300 mM NaOH and 10 mM EDTA, pH 13) and added to an electrophoresis tank. The slides were placed in the electrophoresis chamber for 40 minutes. Following this procedure, the slides were neutralized in Tris-HCl buffer (pH 7.5) for 20 minutes, then rinsed in Tris-HCl buffer for an additional 20 minutes at 4°C, and stained with ethidium bromide. Comet assay photographs captured by the Gel Doc system were quantified using ImageJ, and data were analyzed using GraphPad Prism software [7]. DNA damage was analyzed with regard to the DNA tail. The key indices of DNA damage in this study were quantified using CometScore software.

Caspase 3/7 Activity Assay

Caspase-3/7 activity was measured according to the kit instructions (Kiazist Life Sciences Company, Iran). The assay utilizes a caspase-3/7-specific substrate, Ac-DEVD-pNA, which is cleaved by active caspase-3/7 to release p-nitroaniline (pNA). Enzymatic activity was quantified by measuring pNA absorbance at 405 nm; higher absorbance indicated greater caspase-3/7 activity.

Histopathological Examination

For histopathological examination, a spleen tissue sample was preserved in 10% buffered formalin solution. The fixed tissue was subsequently embedded in paraffin and sectioned. Following deparaffinization and rehydration, the sections were stained with hematoxylin and eosin and examined under an Olympus CX41 light microscope.

Statistical Analysis

The results were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Results are presented as mean $\pm$ standard deviation (SD) of at least three independent replicates. Statistical significance was set at $P < 0.05$.

Results

Comet Assay Analysis of DNA Damage

As shown in Figure 1, MMS administration significantly increased DNA tail length, indicating substantial DNA damage ($P < 0.05$). Treatment with CGA alone at 40 mg/kg did not induce DNA damage, and tail lengths were comparable to those of the negative control. Co-treatment with CGA at 10 and 20 mg/kg conferred significant protection against MMS-induced damage ($P < 0.05$ and $P < 0.001$, respectively).

Caspase-3/7 Activity Assay

As shown in Figure 2, MMS administration resulted in a significant increase in caspase-3/7 activity compared to the NS control group ($P < 0.001$), indicating the induction of apoptosis. In contrast, treatment with CGA alone (40 mg/kg) yielded caspase-3/7 activity levels comparable to the negative control, suggesting that CGA does not inherently activate the apoptotic pathway. Co-treatment with CGA modulated the MMS-induced apoptotic response in a dose-dependent manner. While the group receiving MMS with 10 mg/kg CGA continued to exhibit elevated caspase-3/7 activity ($P < 0.01$), a marked reduction in enzyme activity was observed at higher CGA concentrations (20 and 40 mg/kg) relative to the MMS-only group ($P < 0.001$ for both comparisons).

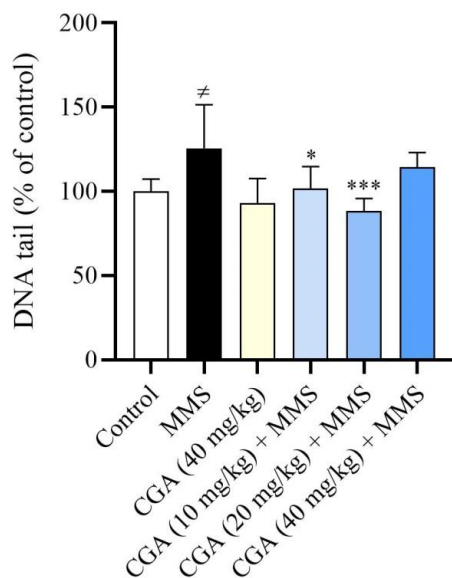


Figure 1. Effect of Chlorogenic Acid (CGA; 10 mg/kg) on DNA Damage Induced by Methyl Methanesulfonate (MMS; 10 mg/kg) in Mouse Spleen.

Statistical analysis used one-way ANOVA with Tukey's test. Values are expressed as means $\pm$ SD, $n=6$ for each group. [#] $P<0.05$, vs control group; ^{*} $P<0.05$, ^{***} $P<0.001$ vs MMS group.

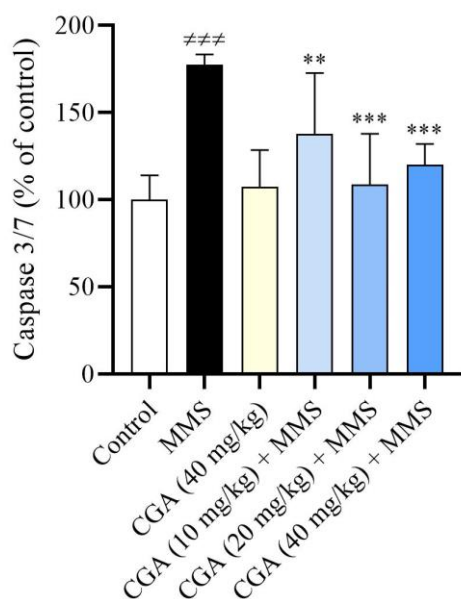


Figure 2. Effect of Chlorogenic Acid (CGA; 10 mg/kg) on Caspase-3/7 Activity in Mouse Spleen

Statistical analysis used one-way ANOVA with Tukey's test. Values are expressed as means $\pm$ SD, $n=6$ for each group. ^{###} $P<0.001$, vs control group; ^{**} $P<0.01$, ^{***} $P<0.001$ vs MMS group.

Histopathological Examination

While the negative control group (NS) exhibited typical splenic architecture with clearly

distinguishable white and red pulp, a normal lymphocyte density, and unremarkable vascular structures, the positive control group treated with MMS alone showed severe tissue disruption. This damage was characterized by decreased clarity, destruction of the white pulp, a marked reduction in lymphocyte density, the presence of irregular structures with increased collagen fibers, and vascular changes accompanied by a relative expansion of the red pulp, confirming the cytotoxic and genotoxic effects of MMS. In contrast, treatment with CGA alone at 40 mg/kg resulted in relatively preserved splenic structure, with distinct cellular white pulp and appropriate lymphocyte density, indicating that CGA alone does not exert destructive effects on tissues. Co-administration of CGA with MMS revealed a clear protective trend. At the lowest dose of 10 mg/kg CGA plus MMS, partial improvement was observed, with some recovery in the white pulp structure and lymphocyte distribution. However, mild impairments persisted in several areas. A more pronounced protective effect was observed in the group treated with MMS plus 20 mg/kg CGA, which showed significant histological improvement compared to the MMS-only group. Key findings at this dose included re-expansion of the white pulp, reduced lymphocyte destruction, diminished fibrotic changes, and a relative return to normal spleen structure, indicating a robust protective and modulatory effect. In the group receiving the highest dose (MMS plus 40 mg/kg CGA), the spleen parenchyma appeared largely normal with well-preserved architecture. However, a noticeable increase in the diameter of some blood vessels was observed, suggesting a vasodilatory effect of the combination of high-concentration CGA and MMS (Figure 3).

Discussion

This study investigated the potential protective effects of CGA against MMS-induced spleen tissue damage in a murine model. Our results demonstrate that MMS exposure induces significant genotoxicity and apoptosis, leading to severe disruption of splenic architecture, and that CGA co-treatment confers a protective effect against this damage.

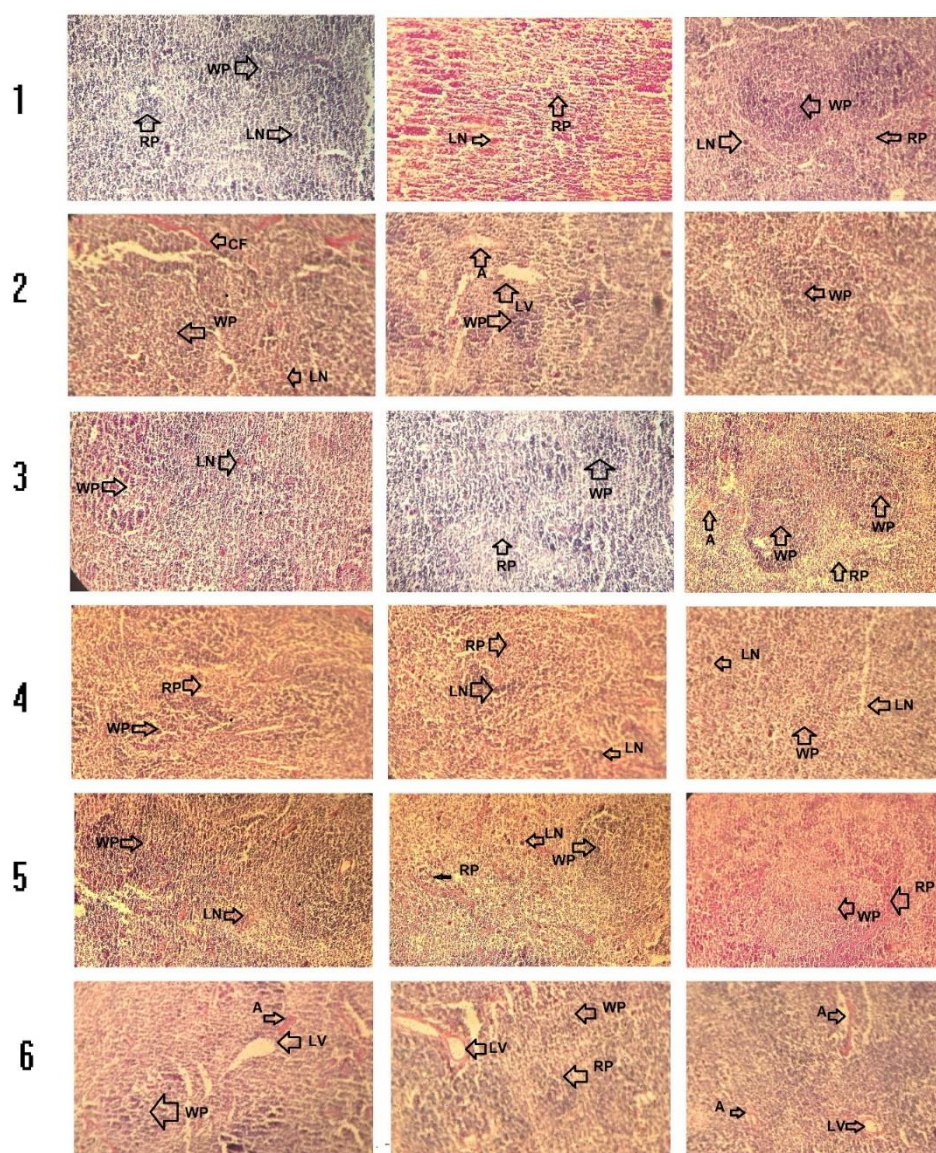


Figure 3. Photomicrographs of Spleen Tissue in Different Groups: (1) A Negative Control Group; (2) Methyl Methanesulfonate (MMS; 10 mg/kg); (3) Chlorogenic Acid (CGA; 40 mg/kg); (4) MMS + CGA (10 mg/kg); (5) MMS + CGA (20 mg/kg); (6) MMS + CGA (40 mg/kg). Original magnification of all images is $\times 40$.

Abbreviations: WP, white pulp; RP, red pulp; CF, collagen fibers; LN, lymphocyte density; A, artery; LN, lymphatic node; LV, lymphatic vein.

Alkaline comet assay results confirmed that MMS, a well-known alkylating agent, caused substantial DNA damage, as evidenced by a significant increase in tail length. This aligns with the established genotoxic mechanism of MMS, which involves the direct alkylation of DNA bases, leading to strand breaks and the generation of apurinic/apyrimidinic sites [6, 7]. Importantly, treatment with CGA alone did not induce DNA damage, indicating its safety at the doses tested. The significant reduction in DNA tail length observed in groups co-treated with CGA

(10 and 20 mg/kg) suggests a potent antigenotoxic effect. This protective mechanism is likely attributed to the well-documented antioxidant properties of CGA. CGA is a potent free radical scavenger that can enhance the activity of endogenous antioxidant enzymes [10], thereby neutralizing the oxidative stress that often accompanies and exacerbates alkylation-induced DNA damage [10, 11]. By mitigating oxidative stress, CGA may reduce the formation of secondary DNA lesions, such as those caused by ROS, thus preserving genomic integrity.

The significant increase in caspase-3/7 activity confirmed the induction of apoptosis by MMS. Caspase-3 and 7 are key executioner caspases in the apoptotic pathway, and their activation is a hallmark of cells committed to programmed cell death [12, 13]. MMS-induced DNA damage is a potent trigger for apoptosis, serving as a mechanism to eliminate cells with potentially mutagenic lesions [6]. The modulation of this apoptotic response by CGA is particularly insightful. At the lowest dose (10 mg/kg), CGA did not prevent the MMS-induced increase in caspase activity, suggesting that although some DNA repair may have been initiated (as evidenced by the comet assay), the damage was sufficient to trigger apoptosis. However, at higher and more protective doses (20 and 40 mg/kg), CGA co-treatment significantly reduced caspase-3/7 activity to near control levels. This finding indicates that at these concentrations, CGA's protective effects extend beyond simple DNA damage prevention to the modulation of cell-survival pathways. By reducing the overall burden of DNA damage, as observed in the comet assay, CGA may enable successful DNA repair, thereby removing the upstream signal required to initiate the intrinsic apoptotic pathway [14]. Furthermore, CGA has been reported to directly modulate pro- and anti-apoptotic proteins, such as Bcl-2 and Bax, potentially raising the cellular threshold for triggering apoptosis [15]. Histopathological examination of the spleen provided a crucial *in vivo* correlation with the molecular findings. The severe disruption of splenic architecture by MMS, characterized by white pulp destruction, lymphocyte depletion, and fibrosis, underscores its potent cytotoxicity in lymphoid organs. The spleen, a secondary lymphoid organ rich in proliferating lymphocytes, is particularly susceptible to genotoxic agents. The preservation of normal splenic structure in the CGA-only group reinforces its safety profile. The progressive histological improvement observed with increasing CGA doses illustrates its protective capacity. The restoration of white pulp cellularity and reduction in fibrotic changes at 20 mg/kg suggest that CGA not only protects lymphocytes from initial DNA damage and apoptosis but also helps preserve the tissue microenvironment and regenerative capacity. This aligns with studies showing that CGA can suppress

inflammatory and fibrotic pathways, potentially by inhibiting nuclear factor kappa-light-chain-enhancer of activated B cells activation and downregulating pro-fibrotic cytokines, such as transforming growth factor-beta [16, 17]. The observation of vasodilation at the highest CGA dose combined with MMS is intriguing and warrants further investigation. CGA is known to have vasodilatory effects, possibly through the modulation of nitric oxide production [18]. This effect may have been potentiated by MMS-induced vascular stress and may represent a specific physiological response rather than a toxic effect.

Conclusion

This study showed that CGA protects mouse spleen tissue from MMS-induced DNA damage, apoptosis, and architectural disruption. CGA shortens DNA tails and reduces caspase-3/7 activity, suggesting enhanced DNA repair and modulation of cell-survival pathways. Histological analysis indicated preserved splenic structure and reduced fibrosis with CGA, indicating anti-inflammatory and anti-fibrotic effects. A vasodilatory response at the highest CGA dose warrants further study to understand its implications for organ perfusion and safety. Future studies should elucidate the molecular mechanisms, assess long-term outcomes, and evaluate CGA across various tissues and under genotoxic conditions.

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Conflict of Interest

The authors declared no conflicts of interest.

Authors' Contributions

Methodology, software, data curation, investigation, validation, funding acquisition, visualization, resources, writing-original draft: MH. P; Conceptualization, methodology, software, data curation, formal analysis, visualization, writing-

review and editing, investigation, and supervision: AM. A;

Conceptualization, methodology, and visualization: M. D;

Conceptualization, methodology, software, data curation, investigation, validation, formal analysis, supervision, visualization, project administration, and writing, review and editing: A. NA.

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