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RESEARCH ARTICLE

Protective Effect of Chlorogenic Acid against Testicular Damage Induced by Glyphosate Isopropilamine in Rats

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ABSTRACT

The aim of this subchronic toxicity study was to determine the prophylactic effect of chlorogenic acid (CGA) on the histopathologic-histologic changes and oxidative stress induced by Glyphosate isopropylamine (GLF-ISO) in testicular tissues. A sum of 42 male Wistar rats were divided into six equal groups, each containing 7 rats. For pretreatment, rats were given CGA at doses of 12.5, 25 and 50 mg/kg (po) and GLF-ISO at 787.85 mg/kg (po) for 7 weeks. GLF-ISO significantly increased malondialdehyde levels while decreasing SOD and CAT activities and GSH levels in testicular tissues. On the contrary, these parameters were improved in CGA-treated groups. Furthermore, CGA ameliorated the histopathological and histological changes in testicular tissues induced by GLF-ISO in a dose-dependent manner. The results indicate that testicular damage caused by GLF-ISO can potentially be prevented or managed by CGA.

Keywords: Glyphosate isopropilamine, Chlorogenic acid, Oxidative stress, Testicular damage

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Sıçanlarda Glifosat İzopropilamin ile Oluşturulan Testiküler Hasara Karşı Klorojenik Asidin Koruyucu Etkisi

ÖZ

Bu subkronik toksisite çalışmasının amacı, testis dokularında Glifosat izopropilamin (GLF-ISO) tarafından indüklenen histopatolojik-histolojik değişiklikler ve oksidatif stres üzerinde klorojenik asidin (CGA) profilaktik etkisini belirlemektir. Toplam 42 erkek Wistar sıçan, her biri 7 sıçan içeren altı eşit gruba ayrılmıştır. Ön muamele için sıçanlara 7 hafta boyunca 12.5, 25 ve 50 mg/kg (po) dozlarında CGA ve 787.85 mg/kg (po) dozunda GLF-ISO verilmiştir. GLF-ISO testis dokularında malondialdehit seviyelerini önemli ölçüde artırırken SOD ve CAT aktivitelerini ve GSH seviyelerini azaltmıştır. Aksine, bu parametreler CGA ile tedavi edilen gruplarda iyileşmiştir. Ayrıca, CGA, GLF-ISO tarafından indüklenen testis dokularındaki histopatolojik ve histolojik değişiklikleri doza bağlı bir şekilde iyileştirmiştir. Sonuçlar, GLF-ISO'nun neden olduğu testis hasarının CGA ile potansiyel olarak önlenebileceğini veya yönetilebileceğini göstermektedir.

Anahtar Kelimeler: Glyphosate İzopropilamin, Klorojenik asit, Oksidatif stres, Testiküler hasar

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INTRODUCTION

Glyphosate isopropylamine (GLF-ISO) is one of the most commonly used formulations of glyphosate, a broad-spectrum herbicide widely employed in agriculture, forestry, industrial weed control, lawns, gardens, and aquatic environments (Hanson 2024, NPIC 2024). Glyphosate itself is an acid molecule and is typically formulated as various salts to facilitate packaging and handling (Wikipedia 2024). Among these salts, the isopropylamine salt is most frequently utilized in commercial herbicidal products, including those produced by Monsanto (NPIC 2024, Wikipedia 2024). This formulation is known for its very low toxicity to rats, with an acute oral LD50 greater than 5000 mg/kg, indicating minimal risk of acute poisoning under normal usage conditions (Turkmen and Dogan 2020). Given its widespread use and low acute toxicity, GLF-ISO remains a critical tool in modern agricultural practices, although its long-term health impacts continue to be a subject of scientific investigation. A recent study (Avdatek et al. 2023) found that long-term exposure to GLF-ISO led to increased oxidative stress, decreased spermatologic parameters and decreased testosterone levels in rat testicular tissue. Previous studies have found that GLF-ISO causes adverse effects on the male reproductive system. Studies have suggested that glyphosate can induce oxidative stress and disrupt endocrine functions, leading to testicular damage. Research indicates that exposure to glyphosate increases the levels of oxidative stress markers and affects sperm integrity, which are critical indicators of reproductive health (Clair et al. 2012, Dai et al. 2016).
Reactive oxygen species (ROS) are vital in many areas of male reproductive function, including spermatozoa's ability to fertilize. However, an increase in ROS formation, caused by internal and external stimuli, can lead to oxidative stress, altering the structure and function of phospholipids and proteins. ROS assault DNA in the nucleus, fragmenting it and activating apoptosis, hence changing gene and protein expression (Juárez-Rojas et al. 2022). Research using animal models to prevent and restore male germinal tissue and function, especially against the oxidative stress effects of herbicides containing glyphosate, has focused on antioxidant compound as a possible technique for preserving male fertility (Avdatek et al. 2023, Avdatek et al. 2018, Hashim et al. 2022, Spadella et al. 2024). According to a recent study, chlorogenic acid (CGA) has the potential to be an important candidate to mitigate GLF-ISO-induced damage in various organs, including the testis and prostate (Jia et al. 2024).
Chlorogenic acids (CGAs) are a family of polyphenolic compounds predominantly found in coffee beans, particularly in green coffee extracts. They have been recognized for their various health benefits, which include antioxidant and anti-inflammatory activities (Tajik et al. 2017). In various in vitro and in vivo studies, CGAs have shown protective effects against oxidative stress, which is a key mechanism involved in many pathological conditions, including testicular damage. CGAs help mitigate oxidative damage by neutralizing free radicals and enhancing the activity of antioxidant enzymes like glutathione peroxidase (Uz et al. 2002).
However, there are no studies on the role of CGA in GLF-ISO-induced testicular damage. In this study, the protective effect of CGA on testicular damage induced by GLF-ISO via gavage in rats was investigated for the first time.

MATERIAL and METHODS

Chemicals

Knock-out, a glyphosate formulation marketed in Turkey by Hektas, was utilized in this investigation. 48% isopropylamine salt is present in the liquid-water soluble formulation of this active ingredient as inert substances and excipients. The selection of this glyphosate-based herbicide was based on the fact that it is one of the most popular herbicides for controlling weeds in Turkey, where it has been shown that eradication is challenging for formulations with high glyphosate levels. CGA (Cat no. C3878, purity ≥ 95%) was purchased from Sigma (Sigma–Aldrich, Shanghai, China).

Ethical statement, Animals and Experimental design

This study's Animal Experiments Local Ethics Committee permission was obtained (49533702-241). Forty-two male Wistar Albino rats with an average age of 2-3 months and weighing 180-200 g were obtained from Afyon Kocatepe University Experimental Animal Research and Application Center. Following one week of adaptation, randomized into six groups (n = 7), the animals were maintained in a controlled environment (22 °C, 12 h light-dark cycle) with free food and water.

Control group: Rats were given 0.5 mL distilled water solution (as chlorogenic acid solvent) per-orally (p.o.) for 49 days.
CGA50: Rats were given CGA (50 mg/kg) dissolved in 0.5 mL distilled water via p.o. route for 49 days.
GLF-ISO group: Rats were given GLF-ISO (LD_{50}/10, 787.85 mg/kg) dissolved in 0.5 mL distilled water via p.o. route for 49 days.
CGA12.5+GLF-ISO: CGA (12.5 mg/kg) was administered via p.o. one hour before GLF-ISO (800 mg/kg) administration and continued for 49 days.
CGA25+GLF-ISO: CGA (25 mg/kg) was administered via p.o. one hour before GLF-ISO (800 mg/kg) administration and continued for 49 days.

CGA50+GLF-ISO: CGA (50 mg/kg) was administered via p.o. one hour before GLF-ISO (800 mg/kg) administration and continued for 49 days.
The dose of GLF-ISO given to the animals was determined using the study by Turkmen and Dogan (2020), and the dose of CGA was determined using the study by Qi et al. (2011).

Sample Collection and Homogenate Preparation
One day after the last drug administration, rats were sacrificed under mild sevoflurane anesthesia. Testicular tissues of sacrificed rats were collected. Right testes were fixed in 10% neutral buffered formalin for histopathologic examination, while the left testes were stored in deep freezer at -20 for tissue biochemical analysis. Testes were taken out of the freezer at -20°C and put straight into the glass tubes to cool. After that, nine times as much phosphate-buffered saline (PBS; pH 7.4) was added to the testes to dilute them. Testes were minced in a glass and homogenized for three minutes in cold physiological saline on ice using a Teflon–glass homogenizer in preparation for the biochemical analyses (Türk et al. 2011).

Measurements of the oxidant-Antioxidant Balance
The method outlined by Ohkawa et al. in (1979) was utilized to quantify malondialdehyde (MDA), while the method reported by Beutler et al. (1963) was employed to assess the concentration of GSH in the tissue homogenates. The techniques outlined by Sun et al. (1988) and Aebi (1984) were utilized to assess the activity of the SOD and CAT antioxidant enzymes in the tissue samples. The researchers utilized the colorimetric technique outlined by Lowry et al. (1951) to quantify the protein concentration in the tissue. The spectrophotometric measurements were conducted using a Shimadzu 1601 UV–VIS spectrophotometer from Tokyo, Japan.

Histopathological evaluations
Testicular specimens from euthanized rats were fixed in 10% neutral buffered formalin for 24 hours, then kept in 80% ethyl alcohol overnight and paraffin blocks were prepared after routine procedures. The 5 micron thick sections taken from the paraffin blocks were stained with the Hematoxylin Eosin method (Luna 1968) and examined under a light microscope and the lesions were recorded. The measurements were performed under X20 objective. Testis lesions were graded semi-quantitatively according to the classifications of Gibson-Corley (2013) as -: no lesion, +: mild, ++: moderate, and +++: severe.

Histomorphometry
Paraffin sections were stained using Periodic Acid Schiff Reagent (PAS) staining method (Culling et al. 1985) to measure the seminiferous tubule diameters (STDs) and seminiferous epithelial heights (SEHs). Four sections were used from each rat. The rat spermatogenesis cycle comprises 14 stages (Hess 1990, Leblond and Clermont 1952); we observed stages VII−VIII of spermatogenesis. Ten round or nearly round stage VII−VIII tubules were selected randomly for each section (Ahbab et al. 2017). The STDs and SEHs were measured in four sections (approximately 40 STDs and SEHs/animal) using an image analysis program (Leica Q-Win Standard, Q-Win Plus 3.5 software, Leica Cambridge Ltd., Cambridge, UK). The measurements were taken at four different regions of each seminiferous tubule in the section.

Statistical evaluation
The SPSS (Version 22.0) statistical program was used for statistical analysis. Data were presented as mean ± standard deviation. In order to evaluate the data, a normality test was applied first. One-way analysis of variance (ANOVA) and the Duncan test for pairwise comparisons were used to determine the differences between groups for data showing normal values. The stage VII−VIII STDs and SEHs were analyzed using Kruskal-Wallis one-way analysis of variance and a post hoc multiple comparison test, the Mann-Whitney U test with Bonferroni correction. In all analyses, a p value less than 0.05 was considered statistically significant.

RESULTS

Effects of CGA on GLF-ISO-induced lipid peroxidation and antioxidant status
The effects of CGA, GLF-ISO, and their combination on testis oxidative stress and antioxidant parameters are presented in Table 1. GLF-ISO significantly increased the levels of MDA in the testis compared to the controls ($p<0.001$). The MDA levels reduced dose-dependently in the testis of rats, which respectively received 12.5, 25 and 50 mg/kg of CGA ($p<0.001$). Also, the oxidant indice did not significantly change due to CGA administration, compared to the control group ($p>0.05$).
Compared to the controls, GSH content, as well as SOD, and CAT activities, significantly reduced in the GLF-ISO group ($p<0.001$). On the other hand, CGA pretreatment at 12.5, 25 and 50 mg/kg for 49 consecutive days caused a dose-dependent increase in the GSH content, besides SOD and CAT activities, compared to the GLF-ISO group ($p<0.001$). Moreover, oxidative stress parameters did not change in normal rats after CGA administration, compared to the controls ($p>0.05$).

Table 1. Effects of glyphosate-isopropilamine(GLF-ISO; 787.85 mg/kg, 10% of the LD_{50}) and three different doses of chlorogenic acid (CGA; 12.5, 25, and 50 mg/kg) on levels of malondialdehyde (MDA) and glutathione (GSH) and activities of superoxide dismutase (SOD) and catalase (CAT) in testis tissue of rats homogenates

Groups	MDA (nmol/g tissue)	GSH (nmol/g tissue)	SOD (U/µg protein)	CAT (k/µg protein)
Control	0.99±0.35^{c}	7.35±0.72^{a}	2.12±0.56^{a}	4.15±0.98^{a}
Chloro50	0.91±0.28^{c}	7.66±0.69^{a}	2.28±0.59^{a}	4.04±0.91^{a}
GLF-ISO	7.26±2.10^{a}	3.54±0.36^{c}	0.89±0.25^{c}	0.94±0.21^{d}
Chloro12.5+GLF-ISO	4.72±2.00^{b}	6.01±0.58^{b}	1.02±0.33^{bc}	1.04±0.41^{d}
Chloro25+GLF-ISO	1.99±0.73^{c}	6.50±0.63^{ab}	1.70±0.48^{b}	1.62±0.35^{c}
Chloro50+GLF-ISO	1.56±0.91^{c}	7.12±0.76^{a}	2.05±0.54^{a}	2.84±0.67^{c}

Note: Mean ± standard deviation; $n = 7$; Values with different letters (a, b, c, d) in the same column are statistically significant ($p < 0.05$)

Effects of CGA on GLF-ISO-Induced Histopathological Changes

Histopathological changes in the testicular tissues of the animals in the experimental groups were described in detail and shown in Figure 1 and Table 2. In the GLF-ISO group, irregular basement membranes, vacuolization and hyalinization in the interstitial area were observed in the seminiferous tubules (Figure 1-A3; Table 2). Few histopathologic changes were observed in GLF-ISO groups (Figure 1-A4-A5-A6; Table 2). Normal tissue was observed in the control group (Figure 1-A1; Table 2) and in the CGA50 group (Figure 1-A2; Table 2).

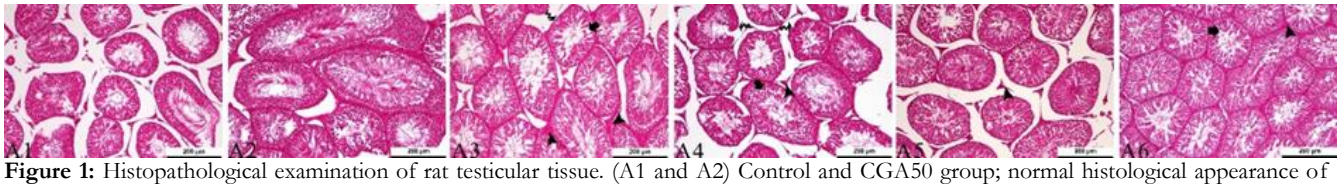

Figure 1: Histopathological examination of rat testicular tissue. (A1 and A2) Control and CGA50 group; normal histological appearance of testicular tissue. (A3) GLF-ISO group; Irregular basement membrane (curved arrow), Vacuolation in the seminiferous tubule (thick arrow) and Hyalinization in the interstitial area (arrowhead). (A4) Chloro12. 5+GLF-ISO group; Irregular basement membrane (curved arrow), Vacuolation in the seminiferous tubule (thick arrow) and Hyalinization in the interstitial area (arrowhead). (A5) Chloro25+GLF-ISO group; Hyalinization in the interstitial area (arrowhead). (A5) (A6) Chloro50+GLF-ISO group; Vacuolation in the seminiferous tubule (thick arrow) and Hyalinization in the interstitial area (arrowhead). All figures were stained with H&E. Original magnifications of 20x and 100 µm were used.

Tablo 2. Semi-quantitative histopathological scoring of testis tissue

Groups	Vacuolation in the seminiferous tubule	Hyalinization in the interstitial area	Irregular basement membrane in the seminiferous tubule
Control	0.00±0.00^{b}	0.00±0.00^{b}	0.00±0.00^{c}
Chloro50	0.00±0.00^{b}	0,00±0.00^{b}	0.00±0.00^{c}
GLF	2.10±0.89^{a}	1.77±0.52^{a}	1.43±0.52^{a}
Chloro12.5+GLF-ISO	1.60±0.54^{a}	0,70±1.08^{a}	0.72±0.87^{b}
Chloro25+GLF-ISO	0.55±0.60^{b}	0,53±0.88^{a}	0.55±0.60^{bc}
Chloro50+GLF-ISO	0.36±0.57^{b}	0.18±0.45^{a}	0.36±0.57^{bc}
p	0.000	0.000	0.001

Note: Mean ± standard deviation; $n = 7$; Values with different letters (a, b, c) in the same column are statistically significant ($p < 0.05$)

Effects of CGA on GLF-ISO-Induced Histomorphometric Changes

Histomorphometric changes in the testicular tissues of the animals in the experimental groups were described in detail and shown in Table 3. In the GLF-ISO group, the STDs at stages VII−VIII were significantly reduced compared to the other groups ($p < 0.001$). The STDs in the CGA12.5+GLF-ISO group were significantly lower than the control group, but significantly higher than the GLF-ISO group. Moreover, there was no significant in the STDs between the CGA25+GLF-ISO and CGAKA50+GLF-ISO groups. The STDs in the CGA25+GLF-ISO and CGA50+GLF-ISO groups were significantly increased compared to the GLF-ISO group. There was no significant in the SEHs at stages VII−VIII between the control and GLF-ISO groups. On the other hand, the SEHs were similar between the CGA25+GLF-ISO and CGA50+GLF-ISO groups.

Table 3. Histomorphometric analysis of the testis in the experimental groups.

Group	n	STDs (µm) $(\bar{X} \pm S_{\bar{X}})$	SEHs (µm) $(\bar{X} \pm S_{\bar{X}})$
Control	7	199,12±0,80[c]	45,53±0,21[c]
CGA50	7	220,56±0,76[a]	55,72±0,22[ab]
GLF-ISO	7	179,99±0,68[e]	45,70±0,20[e]
CGA12.5+GLF-ISO	7	186,59±0,58[d]	50,52±0,14[d]
CGA25+GLF-ISO	7	198,49±0,70[bc]	51,53±0,10[c]
CGA50+GLF-ISO	7	202,72±0,8[b]	56,27±0,22[ac]
p		***	***

[a,b,c,d,e]Means within each grouping with different letter designations differ significantly.
CGA: Chlorogenic acid, GLF-ISO: Glyphosate isopropylamine, STDs: Seminiferous Tubule Diameters, SEH: Seminiferous Epithelium Heights, n: No of rats, $\bar{x}$: Mean, $S_{\bar{x}}$: Standard Error of Mean (SEM).
***: p<0.001.

DISCUSSION

Chronic exposure to glyphosate and glyphosate-based herbicides (GBHs) has been a subject of increasing scrutiny due to potential adverse effects on male reproductive health. Studies have demonstrated that exposure to glyphosate can affect various aspects of the testicular function and spermatogenesis (Liu et al. 2022, Owagboriaye et al. 2017). In this study, we tried to investigate the protective effect of different doses of CGA against testicular damage due to long-term exposure to GLF-ISO in terms of oxidative stress, histological and histopathological aspects.

Oxidative stress is the critical factor in the effect of glyphosate on testicular cells. Oxidative stress results from an imbalance between the production of reactive oxygen species (ROS) and the body's ability to detoxify these reactive intermediates or repair the resulting damage. Several studies have highlighted the role of oxidative stress in male infertility and testicular dysfunction (Pavuluri et al. 2024). For instance, Sharma et al. (2013) noted that oxidative stress is a significant factor in male infertility, with high levels of ROS leading to lipid peroxidation and DNA damage in sperm cells. Moreover, glyphosate (GLF) exposure has been shown to significantly elevate levels of serum MDA and testicular ROS, while decreasing the activity of critical antioxidant enzymes such as CAT, and SOD (Bhardwaj et al. 2022). Such changes are indicative of oxidative damage and have been implicated in the disruption of testicular function. Recent research has highlighted the potential therapeutic role of antioxidants in ameliorating oxidative stress-induced testicular damage. For example, administration of antioxidants such as N-acetylcysteine (NAC) has been shown to reverse oxidative damage in GLF-treated testicular tissues by decreasing lipid peroxidation and enhancing the activity of antioxidant enzymes (Hashim et al. 2022). To counteract the oxidative damage induced by glyphosate, antioxidants have been suggested as potential mitigators. Studies have demonstrated the ameliorative effects of compounds like resveratrol and proanthocyanidin on testicular oxidative stress and DNA damage in rats exposed to GBHs (Avdatek et al. 2023, Avdatek et al. 2018). Similar to these studies, in our study, it was observed that MDA levels in testicular tissue were high, GSH levels and SOD and CAT activities were decreased in GLF-ISO-treated rats. The high MDA levels and low GSH levels, SOD and CAT activities in the GLF-ISO group may be related with the depletion of these enzymes due to the increase in oxidative stress. Compared to GLF-ISO group, MDA levels decreased dose-dependently in GLF-ISO groups treated with CGA and GSH levels, SOD and CAT activities increased significantly in testicular tissue. This suggests that CGA may have the ability to maintain and renew the activity of these enzymes.

Histopathologic and histologic analyses are crucial endpoints for assessing testicular toxicity, providing detailed information on structural and functional changes in the male reproductive system. In the context of glyphosate exposure, evaluation of testicular histopathology reveals important alterations that may affect spermatogenesis and overall testicular health. According to histopathologic examination and quantitative evaluation, GLF-ISO caused significant changes in the testis. Histopathologic and histologic analyses are crucial endpoints for assessing testicular toxicity, providing detailed information on structural and functional changes in the male reproductive system. In the context of glyphosate exposure, assessment of testicular histopathology reveals important changes that may affect spermatogenesis and overall testicular health. According to histopathologic examination and quantitative assessment, GLF-ISO caused significant changes in the testis. In the GLF-ISO group, irregular basement membrane and vacuolization formations in the testicular seminiferous tubule and hyalinization in the interstitial area were observed compared to the control and CGA50 groups. It was concluded that these histopathological disorders decreased in a dose-dependent manner with CGA administration. Avdatek et al. (2018) reported a decrease in sperm concentration and degeneration of Sertoli cells in the testis after administration of Knockdown 48 SL, a

commercial brand of GIS-ISO, to rats at a dose of 375 mg/kg for 8 weeks. In the same study, the authors concluded that resveratrol given as a protective agent ameliorated the histopathological changes caused by GIS-ISO. Nardi et al. (2017) showed that GLF treatment in rats decreased STDs. Similarly, we found that GLF-ISO administration reduced significantly STDs at stages VII−VIII. By contrast, the CGA12.5, CGA25, and CGA50+GLF-ISO treatments alleviated the adverse effects of GLF-ISO on STDs at stages VII−VIII of spermatogenesis. However, we found no difference in SEHs at stages VII−VIII among control and GLF-ISO groups. This could be due to the dose level and the method treatment of GLF-ISO.

CONCLUSION

In this study, CGA was shown to improve oxidative stress parameters and reduce histopathological damage in rats exposed to GLF-ISO-induced testicular damage in a dose-dependent manner, suggesting its potential as a protective agent against oxidative stress in reproductive organs. Further research is required to explore the long-term efficacy of these interventions and their applicability to human populations exposed to glyphosate.

Conflict of interest: The authors have no conflicts of interest to report.

Authors' Contributions: RT and YOB contributed to the project idea, design and execution of the study. OA, HHD, TT and OG contributed to the acquisition of data. RT, OA, HHD and TT analysed the data. RT and OG drafted and wrote the manuscript. YOB, OA, TT and OG reviewed the manuscript critically. All authors have read and approved the finalized manuscript.

Ethical approval: This study was carried out at Afyon Kocatepe University Reserch Animals Application Center. This research was approved by The Ethics Committee of the Faculty of Veterinary Medicine, Afyon Kocatepe University (AKUHADYEK, Ref No: 49533702/241)

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