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Resveratrol's Antiapoptotic and Antioxidant Role in Carbon Tetrachloride-Induced Nephrotoxicity of Rats

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ABSTRACT This study investigates the protective effect of Resveratrol on renal tissues utilizing histopathology, immunohistochemistry, and immunofluorescence methods in a carbon tetrachloride (CCl_4)-induced nephrotoxicity model. Wistar albinos (male, n=32) were randomly selected and divided into groups. The initial control group, followed by the second group, received CCl_4 (2mg/kg), the third group was administered Resveratrol (30 mg/kg/day), and the fourth group was co-administered with CCl_4 and Resveratrol. After the experiment, rats were euthanized under anesthesia, and their kidneys were collected. Results revealed that in the CCl_4 group, notable degeneration and necrosis of tubular epithelium, significant steatosis, and arterial hyperemia wereobserved. In the CCl_4+Resveratrol group, renal tissues exhibited slight deterioration of tubular epithelium and hyperemia in the glomerular arteries. The immunohistochemistry approach employing 8- Hydroxy-deoxyguanosine (8-OHdG) was utilized to identify DNA damage in kidney tissue. Immunohistochemistry results indicated that both the control and resveratrol groups had a negative reaction for 8-OHdG, but the CCl_4 group demonstrated a statistically significant increase in 8-OHdG expression in the tubular epithelium located cytoplasmically ($p<0.05$). Modest cytoplasmic presence was detected in the CCl_4+Resveratrol group. The immunofluorescence approach utilizing caspase 3 was employed to identify apoptosis in kidney tissue. The results indicated that the CCl_4 group demonstrated a statistically significant increase in intracytoplasmic caspase 3 expression in the tubular epithelium compared to other groups ($p<0.05$) while a minor intracytoplasmic response for caspase 3 was observed in the CCl_4+Resveratrol group. As a conclusion, resveratrol demonstrated strong antioxidant, antiapoptotic, and DNA-protective effects in a CCl-4-induced nephrotoxicity rat model and can be added to the daily diet for better kidney health against CCl-4-induced nephrotoxicity.

Keywords: *Antioxidant, Caspase 3, CCl_4, Resveratrol, 8-OHdG.*

ÖZ

Ratlarda Karbon Tetraklorür ile Oluşturulan Nefrotoksisitede Resveratrol'ün Antiapoptotik ve Antioksidan Rolü

Bu çalışma, karbon tetraklorür (CCl_4) kaynaklı nefrotoksisite modelinde histopatoloji, immünohistokimya ve immünofloresan metotlarını kullanarak güçlü bir antioksidan olan Resveratrol'ün böbrek dokuları üzerindeki koruyucu etkisini incelemeyi amaçlamaktadır. Rastgele seçilen erkek Wistar sıçanları (n=32) dört gruba ayrıldı. Kontrol grubuna herhangi bir işlem uygulanmazken, ikinci gruba CCl_4 (2 mg/kg), ve üçüncü gruba Resveratrol (30 mg/kg/gün) verildi ve dördüncü gruba CCl_4 ve Resveratrol birlikte verildi. Deneyden sonra sıçanlara anestezi altında ötenazi uygulandı ve böbrek örnekleri toplandı. CCl_4 grubunda histopatolojik olarak, tübüler epitelyumda belirgin dejenerasyon ve nekroz, hem glomerüler hem de interstisyel bölgede yer alan arterlerde belirgin steatoz ve hiperemi görüldü. CCl_4+Resveratrol grubunda böbrek dokuları tübüler epitelde hafif bozulma ve glomerüler ve interstisyel alanların arterlerinde hiperemi gösterdi. Böbrek dokusunda DNA hasarını belirlemek için 8-OHdG antikoru ile immünohistokimyasal boyama yapıldı. İmmünohistokimya sonuçları hem kontrol hem de resveratrol gruplarında 8-OHdG antikoruna karşı bir reaksiyon oluşmadığını, ancak CCl_4 grubuna ait tübüler epitelde sitoplazmik 8-OHdG ekspresyonunda istatistiksel olarak anlamlı bir artış görüldüğünü ortaya koydu ($p<0.05$). CCl_4+Resveratrol grubunda hafif intrasitoplazmik tutulum tespit edildi. Böbrek dokusunda apoptozu belirlemek için kaspaz 3 antikoru ile immünofloresan boyaması yapıldı. Sonuçlar, CCl_4 grubunun tübüler epitelde diğer gruplara kıyasla intrasitoplazmik kaspaz 3 ekspresyonunda istatistiksel olarak anlamlı bir artış gösterdiğini ($p<0.05$) ortaya koyarken, CCl_4+Resveratrol grubundakaspaz 3'e karşı oluşan yanıt minör ve intrasitoplazmik özellikteydi. Sonuç olarak, resveratrol, CCl-4 kaynaklı nefrotoksisite sıçan modelinde güçlü antioksidan, antiapoptotik ve DNA koruyucu etkiler göstermiştir ve CCl-4 kaynaklı nefrotoksisiteye karşı daha iyi böbrek sağlığı için günlük diyete eklenebilir.

Anahtar Kelimeler: *Antioksidan, CCl_4, Kaspaz 3, Resveratrol, 8-OHdG.*

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INTRODUCTION

Grain fumigation, fire extinguisher filling, and the dry cleaning industry all employ the hazardous chemical carbon tetrachloride (CCl_4) (Unsal et al. 2021). The vast majority of CCl_4 exposure takes place in laboratories and the chemical industry. Numerous investigations have shown that CCl_4 causes kidney damage. Furthermore, the nephrotoxic mechanism of CCl_4 entails cellular injury resulting from oxidative stress induced by an elevation in reactive oxygen species (ROS) (Ozdemir et al. 2022). Oxidative damage is identified by quantifying metabolites such as malondialdehyde (MDA) and 8-hydroxyguanosine (8-OHdG), which are generated due to oxidative harm to macromolecules caused by free radicals. Guanine possesses the lowest ionization potential among DNA constituents and is the most susceptible to oxidation, and 8-OHdG is a mutagen generated in DNA by endogenous reactive oxygen species produced during normal oxidative metabolism or by exogenous reactive oxygen species (Atmaca and Aksoy 2009).

Apoptosis is the regulated demise of cells, governed by genes, to preserve homeostasis in the organism (Green 2005). It encompasses the activation, expression, and control of many genes. Apoptosis occurs via two primary pathways: the extrinsic pathway, activated by apoptosis receptors like TNF receptor 1, and the intrinsic pathway, which commences with permeabilization of the mitochondrial outer membrane, leading to the release of cytochrome C from the intermembrane gap of the mitochondria (Marino et al. 2014). The intrinsic route is intricately associated with mitochondrial energy metabolism. Oxidative stress occurs when the equilibrium between oxidation and antioxidation is disturbed, favoring oxidation and the principal origin of ROS is the mitochondrial respiratory chain (Wu et al. 2017). Failure of the body's antioxidants to neutralize excessive superoxide generation can result in oxidative stress damage and mitochondrial malfunction, ultimately leading to apoptosis. Caspase-3, a member of the cysteine protease family, is regarded as a crucial effector enzyme in the induction of cell death (Wangand Ye 2021). The elevation of free radicals caused by the use of CCl_4 is recognized to induce apoptosis in renal tissues (Emam et al. 2020). Preventive, supplementary, or conventional treatments primarily seek to eradicate oxidative damage and create an environment conducive to cellular repair. Consequently, compounds possessing antioxidant capabilities are predominantly utilized. One of these compounds widely used is resveratrol. The antioxidant activity of resveratrol is contingent upon the configuration of functional groups within its core structure. Consequently, the structure, substitution, and total quantity of hydroxyl groups substantially influence many mechanisms of antioxidant activity, encompassing radical scavenging and metal ion chelation abilities. Prior research indicated that the hydroxyl group at the 4′ position is not the exclusive driver of antioxidant activity; the 3- and 5-OH groups also contribute significantly (Gülçin 2010; Iuga et al. 2012). Resveratrol's antioxidant qualities have effectively been utilized to safeguard cells from oxidative stress induced by hydrogen peroxide, with pre-treatment enhancing cell survival and providing protection against UV irradiation-induced cell death. Resveratrol's cellular defense may be partially attributed to its capacity as a direct antioxidant and as an inducer of indirect cellular antioxidant systems through the modulation of several cellular antioxidant pathways, thus maintaining cellular redox equilibrium (Marques et al. 2009; Konyalioglu et al. 2013; Means et al. 2017).

This studyaimed to illustrate the therapeutic efficacy of resveratrol against CCl_4-induced nephrotoxicity using immunohistochemical analysis utilizing 8-OHdG and immunofluorescent techniques employing Caspase-3.

MATERIAL AND METHODS

All the authors ensure that they protect animal rights in their work and this study was carried out by the ethical rules with the permission decision numbered 75296309-050.01.04-E.1700068324 and numbered 11 by the Atatürk University Animal Experiments Local Ethics Committee.

Experimental Procedure

In the study, approximately 7-8 weeks old 32 male Wistar Albino rats were used. The initial body weight of the rats was approximately 250-300 g. The subjects were randomly divided into 4 groups, each with 8 rats.

Group 1 control (CONT) group (n=8): Drinking water was given for 14 days

Group 2 CCl_4 (CCl_4) group (n=8): In this group, rats were administered a single dose of CCl_4 2 ml/kg (Tekeli and Bildik. 2016) (Sigma, St. Louis, MO, USA) intraperitoneally (suspended with olive oil at a ratio of 1:1) on the first day.

Group 3 Resveratrol (RES) group (n=8): In this group, rats were administered Resveratrol (Solgar, Inc. USA) orally at a dose of 30 mg/kg/day for 14 days (Darwish et al. 2018).

Group 4 CCl_4+ Resveratrol (CCL_4 +RES) group (n=8): In this group, rats were administered a single dose of CCl_4 2 ml/kg/day intraperitoneally (suspended with olive oil at a ratio of 1:1) on the first day and Resveratrol orally at a dose of 30 mg/kg/day for 14 days.

The rats were maintained on a12-hour dark/light cycle during the 14-day experimental period and were housed in cages with standard rat food and tap water *ad libitum* in rooms set at 22±2 °C. At the end of the experiment, the animals were sacrificed under anesthesia, and tissue samples were taken into 10% formalin solution for histopathological analysis.

Histopathological Examination

Upon completion of the evaluation, the tissue samples were preserved in a 10% formaldehyde and standard tissue processing protocols were followed. Sections of 4 µm in thickness were obtained, stained with hematoxylin-eosin (HE) and viewed using a light microscope (Olympus BX 51, JAPAN). The sections were assessed based on their histological characteristics and subjected to statistical analysis.

Immunohistochemical Examination

Tissue sections on poly-L-lysine coated slides were deparaffinized. Subsequently, endogenous peroxidase was inactivated by incubating in 3% H_2O_2. The tissues were subsequently heated in a 1% antigen retrieval solution (citrate buffer, pH 6.1). The slices were treated with a protein block for 5 minutes to avert nonspecific staining in the tissues. The primary antibody (8-OHdG Cat No: sc66036, Dilution Ratio: 1/100, US) was applied to the tissues and incubated. 3-3' Diaminobenzidine (DAB) was utilized as a chromogen in the tissues. The slides were analyzed using a light microscope (Zeiss AXIO GERMANY).

Immunofluorescent Examination

Tissue sections for immunofluorescence analysis were affixed to poly-L-lysine slides, followed by deparaffinization and dehydration. Subsequently,

endogenous peroxidase was inactivated by incubation in 3% H_2O_2. The tissues were subsequently heated in a 1% antigen retrieval solution (citrate buffer, pH 6.1). The slices were treated with a protein block for a few minutes to avert nonspecific staining. The primary antibody (Caspase-3 Cat No: sc56053, Dilution Ratio: 1/100, US) was applied to the tissues and incubated. A secondary antibody for immunofluorescence was utilized as a secondary marker (FITC Cat No: ab6785). Diluted 1:1000, maintained in darkness for 45 minutes. DAPI with mounting media (Cat No: D1306, Dilution Ratio: 1/200 UK) was applied to the sections and incubated in the dark, after which the sections were covered. A fluorescent microscope was used to analyse the tissues (Zeiss AXIO GERMANY). The intensity of antibody positivity in immunofluorescence stainings was measured using the ImageJ software (Bolat et al. 2024).

Statistical Analyses

Histopathological investigations utilized the GraphPad Prism 8.0.2 software for statistical analysis, with $p<0.05$ being significant for data evaluation. The Duncan test was employed for group comparison. The non-parametric Kruskal-Wallis test was employed to assess group interactions, while the Mann-Whitney U test was utilized to identify differences between groups. To assess the intensity of positive staining from the images produced by immunohistochemistry and immunofluorescence staining, five random areas were picked from each image and analyzed using ZEISS Zen Imaging Software. Data were statistically represented as mean and standard deviation (mean±SD) for area percentage. A one-way ANOVA, followed by a Tukey test, was conducted to compare positive immunoreactive cells and immunopositive stained regions with healthy controls. The test yielded a $p<0.05$ value, deemed significant, and data were expressed as mean±SD. W/sr (Luminous Intensity Unit). Values denote mean±standard error (n=5). Values with distinct superscripts from the control, denoted as 'a' across columns, are substantially different ($p<0.05$).

RESULTS

Histopathological Results

CONT group: The histopathology examination of the kidney tissues revealed a normal histological appearance (Figure 1 and 2).

CCl_4 group: Histopathological analysis of renal tissues demonstrated significant degeneration and necrosis of tubular epithelium, pronounced steatosis, and hyperemia of arteries within glomerular and interstitial compartments (Figure 1 and 2).

RES group: The histopathological analysis of the renal tissues demonstrated a normal histological appearance (Figure 1 and 2).

CCl_4+RES group: Histopathological analysis of renal tissues demonstrated modest degradation of tubular epithelium and hyperemia in arteries within glomerular and interstitial regions (Figure 1 and 2).

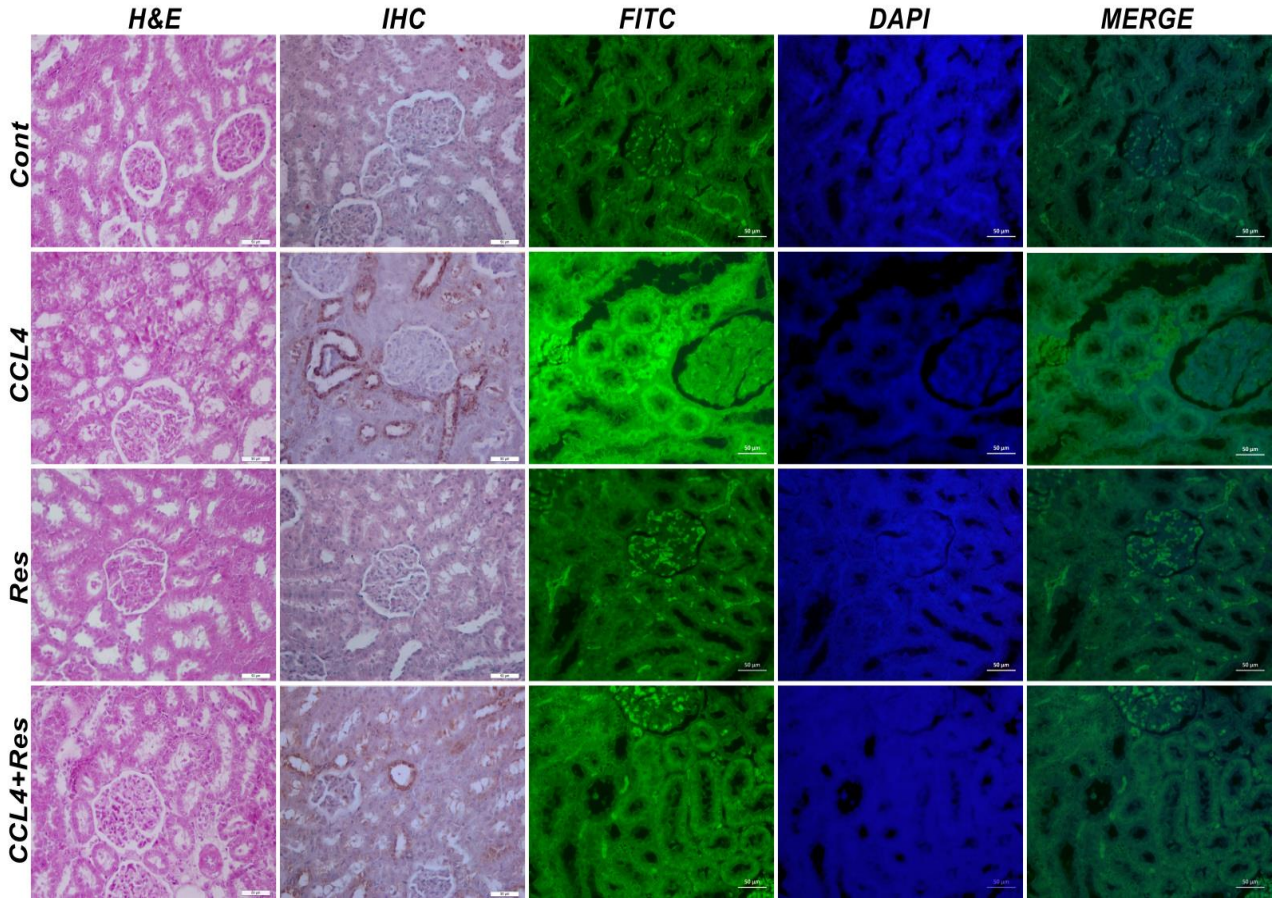

Figure 1: Kidney tissue. Histopathological, immunohistochemical and immunoflourescant findings in kidney tissues, H&E, 8-OHdG, FITC, DAPI, MERGEIHC-P, Bar: 70 µm.

Immunohistochemical Results

CONT group: Immunohistochemical examination of kidney tissues revealed negative expression of 8-OHdG (Figure 1 and 2).

CCl_4 group: Immunohistochemical examination of kidney tissues revealed elevated cytoplasmic 8-OHdG expression in the tubular epithelium (Figure 1 and 2).

RES group: Immunohistochemical examination of kidney tissues revealed negative expression of 8-OHdG (Figure 1 and 2).

CCl_4+RES group: Immunohistochemical staining of renal tissues revealed the modest cytoplasmic presence of 8-OHdG in the tubular epithelium (Figure 1 and 2).

Immunofluorescence Results

CONT group: Immunofluorescence analysis of kidney tissues revealed negative expression of caspase 3 (Figure 1 and 2).

CCl_4 group: Immunofluorescence analysis of kidney tissues revealed pronounced intracytoplasmic caspase 3 expression in the tubular epithelium (Figure 1 and 2).

RES group: The examination of kidney tissues using the immunofluorescence technique revealed a negative expression of caspase 3 (Figure 1 and 2).

CCl_4+RES group: Immunohistochemical examination of kidney tissues revealed modest intracytoplasmic caspase 3 expression in the tubular epithelium (Figure 1 and 2).

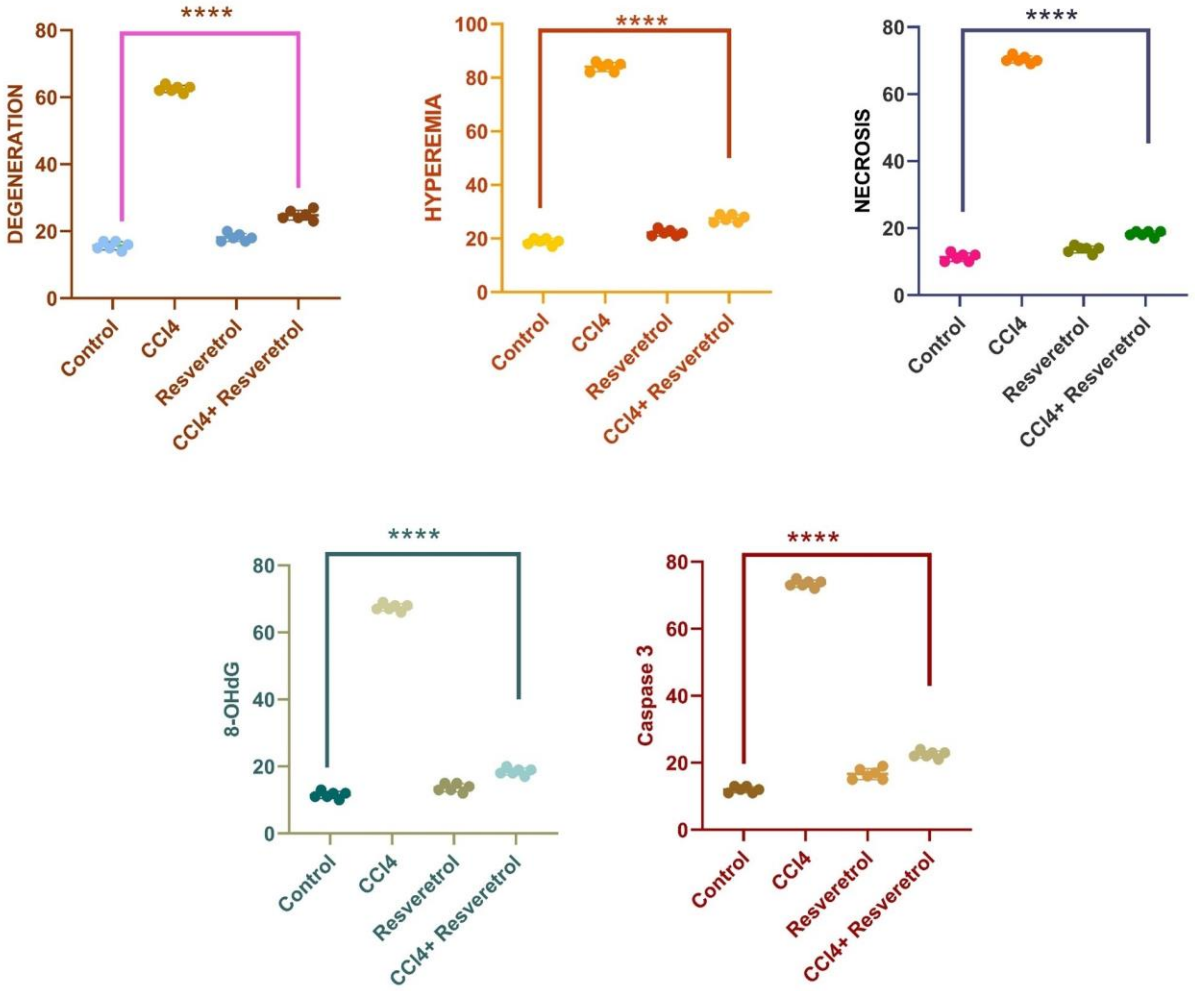

Figure 1: The statistical calculation of the groups for degeneration, hyperemia, necrosis, 8-OHdG and Caspase-3.

DISCUSSION AND CONCLUSION

The current study investigated the possible therapeutic impact of RES on the CCl_4-induced nephrotoxic model in rats. Frequently, the liver and gastrointestinal system are the initial organs that come to mind when detoxification is referenced. While these two systems significantly eliminate toxins from the body, the kidneys are another essential organ involved in detoxification. The kidneys can perform substantial oxidation, reduction, hydrolysis, and conjugation reactions (Lash 1994; Kieffer and et al. 2016).

The kidney's elevated metabolic rate renders it very susceptible to oxidative damage, and numerous studies have demonstrated that oxidative stress can induce or exacerbate disease progression and consequences (Pellegrino and et al. 2019). Recent findings have confirmed that CCl_4 treatment may cause disruptions in the oxidant/antioxidant balance, leading to elevated ROS and subsequent oxidative stress (Xu and et al. 2022). Oxidative stress is the condition in which the balance that exists between antioxidants and oxidants is skewed in favor of the oxidants. Oxidative stress-induced reactive

oxygen species (ROS) production is highly reactive and oxidizes DNA, resulting in DNA damage (Jan and Khan 2016). The metabolic activation of CCl_4 by cytochrome P450 produces free radicals, notably trichloromethyl and trichloromethyl peroxy radicals, which are recognized for intensifying lipid peroxidation and protein oxidation, resulting in significant membrane damage (Andritoiu et al. 2014). CCl_4's nephrotoxic effects have been associated with the production of these free radicals, as indicated by various studies. In the studies, CCl_4-induced nephrotoxicity was found to cause DNA damage and apoptosis (Andritoiu et al. 2014; Jan and Khan 2016; Saif et al. 2024). In some other studies, comet assay was used to demonstrate DNA damage induced in the kidney by CCl_4 (Fahmy et al. 2018; Pegoraro et al. 2021), and was found to be increasing. 8-OHdG is a significant oxidative DNA lesion resulting from the oxidation of the C-8 position of 2′-deoxyguanosine, frequently utilized as a biomarker for oxidative DNA damage. In one of the studies performed on mice, the increased levels of 8-OHdG were revealed to determine kidney DNA damage in the CCl_4-treated mice (Ma et al. 2014). This study uniquely demonstrates DNA damage in CCl_4-induced nephrotoxicity immunohistochemically utilizing the 8-OHdG antibody, a finding not previously reported in the literature. Despite employing a different methodology, our findings corroborated the previous literature indicating an elevation in DNA damage within the CCl_4 groups.

Apoptosis is a meticulously regulated mechanism that dictates cellular fate in responseto diverse stressors. DNA damage is a significant stressor that not only initiates repair mechanisms but also plays a crucial role in regulating cell destiny (Nowsheen and Yang 2012). The endoplasmic reticulum of the kidney's proximal tubule cells contains CYP2E1 enzymes that convert CCl_4 into highly reactive trichloromethyl (CCl_3) and trichloromethyl peroxide radicals and CCl_4 enhances the activity of critical apoptotic enzymes by the rise in the oxidative stress levels. Caspases are essential mediators of apoptosis. Caspase-3 is a commonly activated apoptotic protease that catalyzes the selective cleavage of numerous essential cellular proteins (Porter and Janicke 1999). It is also essential for some characteristic features of apoptosis and is crucial for apoptotic chromatin condensation and DNA fragmentation across all investigated cell types. Our investigation established a correlation between CCl_4-induced oxidative renal damage and caspase 3 immunoreactive apoptotic cells within the kidney. We speculate that the rise in apoptotic cell count is directly associated with CCl_4-induced renal toxicity, heightened oxidative stress, and DNA damage. Research in the literature also indicates that CCl_4-induced renal injury leads to apoptosis (Ma et al. 2018; Safhi 2018; Shaban et al. 2021; Ozdemir et al. 2022,). To our knowledge, the presentation of caspase 3 immunoreactive cells in the CCl_4-induced nephrotoxicity using the immunofluorescence method is the first time employed by this research.

Necrosis, degeneration, and hyperemia are the primary indications of the kidney's natural structure disruption. CCl_4 is a powerful environmental nephrotoxin that induces steatosis, necrosis, and cirrhosis and it acts through hepatorenal syndrome or direct renal tubular damage, specifically necrosis of the proximal tubule and loop of Henle (Prakash et al. 2003; Cooksey 2012). The metabolites of CCl_4 are free radicals, which are likely responsible for this cell necrosis. Oxidative stress is a significant pathophysiological mechanism that contributes to kidney injury, and it can be triggered by exposure to toxins, like CCl_4. Lipid peroxidation causes cellular damage and inflammation, while oxidative stress disrupts the endothelial relaxant nitric oxide, leading to vasoconstriction, and platelet adhesion. Our results presented significant degeneration and necrosis of tubular epithelium, pronounced steatosis, and hyperemia of arteries within glomerular and interstitial compartments in the CCl_4 group, and are in accordancewith the previous studies. Micromorphological injury to the kidney unequivocally results in structural and, eventually, functional decline, underscoring the significance of maintaining the form-function link and requiring detailed investigation.

Plant-derived natural antioxidants are demonstrated to be highly effective in mitigating toxicities and stress conditions induced by CCl_4 radicals in renal injuries. One of these natural antioxidants is resveratrol. Resveratrol is a naturally occurring chemical substance, classified as a polyphenol, synthesized by certain plants in responseto various detrimental stimuli, including pathogen invasion, ultraviolet radiation, or heightened oxidative stress. An overabundance of reactive oxygen species (ROS) is implicated in various illnesses, the aging process, and multiple cellular response pathways. Reactive oxygen species encompass superoxide ($O2^-$), the hydroxyl radical ($OH\cdot$), and peroxynitrite ($ONOO^-$), which target biological proteins and DNA. Oxidative stress arises from an imbalance between reactive oxygen species (ROS) generation and antioxidant defenses; hence, the administration of exogenous antioxidants or the modulation of antioxidant enzymes may mitigate oxidative stress. Prior research has demonstrated that resveratrol can directly neutralize reactive oxygen species (ROS), including $O2^-$, $OH\cdot$, and $ONOO^-$. Our findings indicated that resveratrol administration in CCl_4-induced renal toxicity yielded beneficial effects on micromorphology, DNA damage, and apoptosis. We believe that the results we obtained are directly linked to the reduction of oxidative stress induced by CCl_4, attributable to the antioxidant properties of resveratrol. Research indicates that resveratrol mitigates renal injury induced by various pharmaceuticals, such as glycerol, gentamicin, and cyclosporine by diminishing oxidative stress, which is one of the mechanisms underlying its renal protective effect.

In conclusion, the findings of this study highlight the potential of resveratrol as a therapeutic agent in the reduction of CCl_4-induced nephrotoxicity. This protective effect is predominantly attributed to the compound's potent antioxidant capacity, which effectively neutralizes ROS and reduces oxidative stress, a significant contributor to kidney injury. Furthermore, the anti-apoptotic properties of resveratrol contributed to the maintenance of renal cellular integrity by modulating apoptotic pathways and reducing cell death. These dual mechanisms not only emphasize the multifaceted nature of the protective effects of resveratrol but also suggest its broader applicability in combating oxidative stress-related renal pathologies.

Future research will further elucidate optimal dosing strategies and potential synergistic effects when combined with other therapeutic agents to enhance renal protection and recovery. However, this study has some limitations. The number of animals used and the induction of resveratrol in a certain dose for a short period are the limitations of this study. In the future, the studies conducted can be based on larger experimental groups with different doses of resveratrol in longer days of experiment.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: SY
Supervision / Consultancy: SY, GÇD
Data Collection and / or Processing: FB, BO
Analysis and / or Interpretation: FB, SY, GÇD
Writing the Article: FB
Critical Review: SY, GÇD

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