Protective effects of naringenin on carbon tetrachloride-induced acute nephrotoxicity in mouse kidney

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Abstract
The ability of naringenin (NGN) to protect the kidney against CCl4-induced renal toxicity in male Swiss mice was investigated. The flavonoid was given orally to mice for 7 days and then on the 8th day, these were intraperitoneally injected with 10 mmol/kg CCl4. When the toxicant was administrated alone, an increase of malondialdehyde (MDA) concentration was observed and a significant decrease in superoxide dismutase (SOD), catalase (CAT) glutathione-peroxidase (Gpx) specific activities as well as glutathione (GSH) levels was detected after 24 h. These were accompanied by glomerular and tubular degenerations, vascular congestion, necrosis and fatty changes. Marked collagen deposition and strong TGF-β1 expression were observed mainly in the mesangial cells of the glomeruli and tubulointerstitial areas. Ultrastructural investigations showed proximal and distal tubular epithelial cells alterations including numerous lysosomes and dense granular bodies, altered mitochondria, appearance of “myeloid bodies” and basal enfolding dilatation. Pre-treatment with NGN resulted in the return of biochemical markers to control values. Histopathological and electron-microscopic examinations confirmed the biochemical results. In conclusion, NGN showed antioxidant and renal protective effects against injuries induced by CCl4.

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1. Introduction
Carbon tetrachloride (CCl4), an industrial solvent, cleaner, and degreaser, has been extensively used in animal models to explore chemical toxin-induced hepatic injury [1]. It was demonstrated that liver is not the only target organ of CCl4 toxicity, and trichloromethyl radicals generation (CCl3OO) also occurred in other tissues, such as the kidney [2–5], lung [6,7], testis [8,9], brain [10,11] and blood [12]. It has also been reported that exposure to CCl4 results in renal injuries [13].

The kidney removes the metabolic waste, the xenobiotics and their metabolites, while also regulating the water and ion content in the blood. Xenobiotics can be excreted unchanged into the urine, bile, faeces, expired air or can be converted through biotransformation processes into more water soluble metabolites. The kidneys possess the common xenobiotic metabolizing enzymes, mainly localized in proximal tubular cells [14]. The initial step in biotransformation of CCl4 is reductive dehalogenation [15]. As electrophils, free radicals initiate the lipid peroxidation process and if this process overwhelms the antioxidant defense system, oxidative stress leading to kidney damage occurs.

Many studies have reported that antioxidants can prevent nephropathy and hepatic damage by counteracting free radicals and preventing lipid peroxidation. Additionally, previous reports have shown that hepatic and renal toxicity caused by CCl4 were diminished by natural products such as vitamin C which restored the normal level of reduced glutathione [16] and melatonin which decreased the elevated TBARS and up-regulated the low activities of antioxidant enzymes [5].

Flavonoids are phenolic compounds widely present in the fruit and vegetables characteristic of the human diet. The former have been suggested to exhibit a powerful antioxidant activity due to their ability to reduce free radical formation and scavange free radicals, together with the up-regulation of antioxidant defenses [17].

Naringenin (NGN) is a bioflavonoid highly enriched in citrus fruit, tomatoes and cocoa. This compound has been investigated for its pharmacological activities, including anti-tumor [18–22], anti-inflammatory [23–25], anti-viral [26,27] and anti-diabetic [28] effects.

As far as we know, there is no study concerning the effect of naringenin against CCl4-induced renal injury. Therefore, our work was carried out to establish the protection of naringenin against...
CCL₄-induced damage in mice kidney. In order to evaluate oxidative stress, the specific activities of SOD, CAT and GPX, as well as GSH and MDA concentrations were determined. The changes in mice renal tissue were also evaluated from a structural and ultrastructural point of view.

2. Materials and methods

2.1. Animals and experimental design

Swiss male mice (25 ± 3 g), supplied by the Animal House of the Vasile Goldis Western University of Arad, were used. The animals were acclimatized at a temperature of 20–25 °C under natural light/dark conditions for two days and were fed with food and water ad libitum. Prior to the experiment, the animals were kept under fasting overnight. All experimental procedures were approved by the ethics and regulations of animal experiments of Vasile Goldis Western University of Arad.

Thirty-two animals were used for the experiment and these were divided into 4 groups, as follows:

Group 1: Control animals; received by gavage only the vehicle (i.e. olive oil and 0.5% carboxymethyl cellulose) every day for 7 days and on 8th day were deprived of food for 24 h.

Group 2: CCL₄ group; received the vehicle (i.e. olive oil and 0.5% carboxymethyl cellulose) every day for 7 days and were subsequently i.p. injected with CCL₄ at a dose of 10 mmol/kg b.w. in 50% olive oil (1:1) on the 8th day.

Group 3: NGN pre-treated group; received naringenin solubilized in 0.5% carboxymethyl cellulose (NGN, Sigma 98%) (50 mg/kg b.w.) orally for 7 days and were subsequently i.p. injected with CCL₄ (10 mmol/kg b.w.) on the 8th day.

Group 4: NGN group; received naringenin (NGN) solution alone (50 mg/kg b.w.) orally for 7 days and on 8th day were deprived of food for 24 h.

After 8 days from the start of treatment of groups 1 and 3, and 24 h after CCL₄ i.p. injection for groups 2 and 3, the mice were sacrificed by cervical dislocation. Kidney samples were used for histopathology, electron microscopy and biochemical analyses.

2.2. Kidney tissue homogenate preparation

Fresly isolated fragments of mice kidneys were fixed in Bouin solution, dehydrated in toluene and embedded in paraffin. Five micrometer thickness kidney sections were deparaffinized and processed routinely for Hematoxylin–Eosin staining, Masson’s trichrome, Oil Red O and periodic acid-Schiff (PAS) reactions.

2.3. Assessment of antioxidant status

Renal catalase (CAT) activity was assessed according to the Aebi method [29] that records the decrease in the absorbance at 240 nm corresponding to H₂O₂ decomposition. One unit of CAT activity was represented by the decomposition of 1 μmol H₂O₂/min/mL. Kidney superoxide dismutase (SOD) activity was measured by the method described by Paoletti and Moccal [30]. One unit of SOD activity was equal to the amount of enzyme that inhibits the oxidation of NADH by 50% at 37 °C. The activity of glutathione peroxidase (GPx) was assayed by monitoring the oxidation of NADPH by t-butyl-hydroperoxide at 340 nm (V–530 JASCO spectrophotometer) [31]. All enzymatic activities were calculated as specific activities (units/mg of protein).

2.4. Assessment of peroxidative stress

The renal malondialdehyde (MDA) content was assayed as a measure of lipid peroxidation using a fluorimetric method described by Del Rio et al [32]. The kidney tissue homogenate sample (200 μL) was incubated with 0.1 M HCl (700 μL) for 20 min at room temperature. After that, 900 μL of 0.025 M thiobarbituric acid was added, and the mixture was incubated for 65 min at 37 °C. Subsequently, samples were subjected to fluorescence analysis (λex = 520 nm; λem = 549 nm) (Spectrofluorometer FP-6300 JASCO). Relative fluorescence units (RFU) recorded were converted to nmol malondialdehyde (MDA) using 1,1,3,3-tetramethoxypropane as standard.

2.5. Protein concentration measurement

The protein content was determined after Lowry’s method using bovine serum albumin as standard [33].

2.6. Histopathology

Fig. 1. Protective effect of the 50 mg/kg dose of NGN on MDA concentration induced by CCL₄ exposure in mice kidneys. Values are expressed as means ± SD (n = 8). ***Statistical significance at p < 0.001 as compared to control. ###Statistical significance at p < 0.001 as compared to CCL₄-treated group.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>SOD U/mg protein</th>
<th>CAT U/mg protein</th>
<th>GPX U/mg protein</th>
<th>GSH nmol/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.85 ± 0.34</td>
<td>0.66 ± 0.12</td>
<td>40.14 ± 4.16</td>
<td>9.53 ± 1.00</td>
</tr>
<tr>
<td>CCL₄</td>
<td>2.02 ± 0.22 ***</td>
<td>0.39 ± 0.03 ***</td>
<td>25.86 ± 4.14 ***</td>
<td>7.02 ± 1.88 ***</td>
</tr>
<tr>
<td>NGN</td>
<td>2.05 ± 0.34</td>
<td>0.68 ± 0.03</td>
<td>41.40 ± 5.81</td>
<td>10.22 ± 1.25</td>
</tr>
<tr>
<td>NGN + CCL₄</td>
<td>2.05 ± 0.24 ***</td>
<td>0.63 ± 0.11 ***</td>
<td>37.21 ± 1.90 ***</td>
<td>10.22 ± 1.41 ***</td>
</tr>
</tbody>
</table>

Note. Values are expressed as means ± SD (n = 8). ***Significance versus control p < 0.001. ###Significance versus CCL₄-treated group p < 0.001.
eosin (H&E) and Mallory trichrome staining according to Bio Optica staining kit. Frozen sections were cut at 8 μm with the SLEE MNT cryotome, fixed in 10% buffered formaldehyde and stained with Oil Red O kit according to the methods of Bio-Optica staining kits. Mounted slides were examined under a light microscope (Olympus BX43 microscope) and photographed using a digital camera Olympus XC30.

2.7. Immunohistochemical determination of TGF-β1

Immunohistochemical studies were performed on paraffin embedded kidney tissues using rabbit polyclonal anti-TGFβ1 antibody diluted 1:50 (Santa Cruz Biotechnology, California, USA). Slides were incubated with a peroxidase block. After washing, monoclonal antibodies diluted in phosphate buffered saline

Fig. 2. Photomicrographs of kidney sections stained with hematoxylin–eosin under the light microscope. (A1) Control mice cortex showing normal renal corpuscles formed of tuff of glomerular capillaries (arrowhead) surrounded by Bowman's space (arrowhead). Normal proximal and distal convoluted tubules are also seen. (A2) Normal aspect of control mice medulla region. (B1) CCl4-treated mice renal cortex showing tubular renal epithelial vacuolization (arrowhead) and glomerular atrophy (arrow). (B2) Dilatation or vascular congestion (arrow) and necrosis (arrowhead) into medulla area of CCl4-treated mice. (C1) NGN + CCl4 group displaying a marked improvement of the renal cortex histological aspect which is comparable to that of the control group. (C2) NGN + CCl4 group showing the normal aspect of the medulla area which is comparable to that of the control group. (D1) NGN alone group showing a normal aspect of the cortex area which is comparable to that of the control group. (D2) NGN alone group showing a normal aspect of the cortex area which is comparable to that of the control group.
supplemented with bovine serum albumin were added to tissue samples and incubated overnight at 4°C in a humid environment, followed by incubation with peroxidase labeled polymer conjugated to secondary antibodies. The immunoreaction product was visualized by adding the substrate-chromogen diaminobenzidine (DAB) solution, resulting in a brownish coloration at antigen sites. Tissues were counterstained with hematoxylin, dehydrated in a gradient of alcohol and mounted.

The specificity of the reaction was confirmed by substitution of primary antibodies with irrelevant immunoglobulins of matched isotype, used in the same conditions and dilutions as primary antibodies. Stained slides were analyzed by light microscopy (Olympus BX43, Tokyo, Japan).

2.8. Immunohistochemical determination of TIM-1

Immunohistochemical studies were performed on paraffin embedded kidney tissues using rabbit polyclonal anti-TIM1 antibody diluted 1:50 (Thermo Scientific, USA). Slides were incubated with rabbit ABC staining system (sc-2018), according to the methods of Santa Cruz staining kits (Santa Cruz Biotechnology, USA). Tissues were counterstained with hematoxylin, dehydrated in a

Fig. 3. Photomicrographs of kidney sections stained with Mallory tricrome under the light microscope. (A) Control mice kidney showing few collagen fibers (arrowhead) surrounding the renal corpuscles and tubules. (B) CCl4-treated mice kidney showing many collagen fibers (arrowhead) around renal corpuscles and tubules. (C) NGN + CCl4 group displaying marked improvement of the renal histological aspect which is comparable to that of the control group. (D) NGN alone group showing the normal aspect of the kidney which is comparable to that of the control group.
gradient of alcohol and mounted. The specificity of the reaction was confirmed by substitution of primary antibodies with blocking solution, used in the same conditions and dilutions as primary antibodies. Stained slides were analyzed by light microscopy (Olympus BX43, Tokyo, Japan).

2.9. Electron microscopy

Electron microscopic kidney specimens were prefixed in 2.7% glutaraldehyde solution in 0.1 M phosphate buffer for 1.5 h, at 4°C. Following this, they were washed in 0.15 M phosphate buffer (pH 7.2) and post-fixed in 2% osmic acid solution in 0.15 M phosphate buffer for 1 h at 4°C. Dehydration was performed in acetone, and inclusion was done in the epoxy embedding resin Epon 812. The blocks were cut with an ultramicrotome type LKB, at 70 nm thickness. The sections were doubly contrasted with solutions of uranyl acetate and lead citrate and analyzed with a TEM Tecnai 12 Biotwin electron microscope.

2.10. Statistical analysis

All results were analyzed and plotted using GraphPad Prism software (Version 5; GraphPad Software, Inc., La Jolla, CA) and expressed as mean values ± SD (n = 8). Comparisons between groups were evaluated by one-way ANOVA followed by a post hoc Bonferroni test. A value of p < 0.05 was considered to be statistically significant.

3. Results and discussion

NGN is generally present in food as its β-glycoside, i.e. naringin, which is deglycosylated prior to intestinal absorption [34]. There are important inter-individual differences in the ability of humans to convert naringin to NGN due to the presence or absence of certain bacterial strains in the gut [35]. Furthermore, human intestinal bacteria can metabolize naringin to NGN and then to 4-hydroxybenzoic acid, phloroglucinol, 2,4,6-trihydroxybenzoic acid and 4-hydroxyphenyl acetic acid [36].

Also, it was noticed that a large percentage of NGN absorbed in humans is conjugated as glucuronide derivates [35] which can be excreted in bile and to a lesser extent in urine.

Depending on the diet type, the human intake of NGN could vary between 2.2 mg/day in Denmark [37], 4.1 mg/day in Japan [38], 8.3 mg/day in Finland [39] and 58.1 mg/day in UK [40]. In our experiments we used a single dose of 50 mg/kg b.w., which was proved to be protective in other toxicant mediated oxidative damage in rodents [41,42].

3.1. Lipid peroxidation and effects on renal antioxidant status

As highlighted in Fig. 1, the exposure to CCl4 increased the level of MDA, which is generated by the lipid peroxidation cascade, by 80%. As one of the end products of this process, MDA reacts with protein amino, sulfhydryl and imidazole groups [43], as well as with DNA bases [44]. Due to its bifunctional aldehydic property, MDA has the potential to cross-link proteins, which can reduce or abolish their function [45]. On the other hand, MDA adducts with DNA bases are mutagenic and carcinogenic [46]. The pretreatment with NGN decreased lipid peroxidation significantly compared to CCl4 group to a level almost identical with the control level. This occurrence might be explained by the capacity of NGN, as other polyphenolic compounds, to scavenge the reactive species [47], by acting as terminators of free radical chains and as chelators of redox-active metal ions that are able to catalyze lipid peroxidation [48].

SOD, CAT and GPX specific activities in the kidney were significantly decreased in CCl4 treated mice in comparison with the control group (Table 1), which is in accordance with other data.

![Fig. 4. Photomicrographs of kidney sections stained with Oil Red O under the light microscope. (A) Control mice kidney showing few lipid drops in renal parenchyma. (B) CCl4-treated mice kidney showing proliferation of lipids (red aspect) in renal parenchyma compared with control. (C) NGN + CCl4 group displaying marked improvement of the renal histological aspect which is comparable to that of the control group. (D) NGN alone group showing a normal aspect of kidney which is comparable to that of the control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)](image-url)
which proved that oxidative stress reduced the aforementioned enzymatic activities [49]. The decrease of these enzymatic activities could be explained by the crosslinking of their molecules caused by the formation of MDA-protein adducts which affects their conformation and biological activity, as well as by the cell death. The NGN + CCl₄ treated group had a significant increase in renal antioxidant enzymes activity when compared to CCl₄ treated mice. These results indicate that the inhibition of antioxidant enzymes activity in kidney intoxicated with CCl₄ was prevented by NGN treatment. Previous studies have reported partial NGN-related protection of the kidney against increased lipid peroxidation and the decline of the antioxidant enzymes system due to cadmium [50], cisplatin [51], arsenic [52] or lead [53] administration in rats.

In the group treated with NGN only, the specific activities of CAT and GPX were at the same level with the control one, whereas an up-regulation of SOD activity by 15% was observed. This might be a consequence of the increased expression of SOD at protein level as previously observed in rats exposed to polyphenols [54,55].
The administration of 50 mg/kg b.w. NGN to mice did not affect the level of GSH compared to the control, suggesting that this natural compound does not affect GSH biosynthesis and/or regeneration (Table 1). Our findings differ from other studies showing either a non-protective in vitro effect on oxidation of GSH [56] or an increase in the level of this non-enzymatic antioxidant in the liver of young rats upon NGN administration [57].

### 3.2. Protective effects of naringenin against nephropathy induced by CCl₄

The kidney sections of the control group showed normal appearance of tubules, glomeruli and tubulointerstitial cells (Fig. 2A1–2). Collagen deposition (Fig. 3A1–2) and lipids (Fig. 4A) were at normal range in the control group.

The group of mice treated with NGN alone preserved normal kidney morphology and architecture (Figs. 2D1–2, 3D1–2 and 4D). Histopathological studies revealed that CCl₄ induced glomerular and tubular degenerations varying from glomerular basement thickening (Figs. 2B1 and 3B1) and mild dilatation to congestion of Bowman's space with glomerular atrophy (Fig. 2B1). In addition, some of the renal tubules were dilated and their epithelial cells tended to be vacuolated (Fig. 2B1). As far as the renal cortex of CCl₄-treated group is concerned, the aforementioned lesions were spread to the subcortical and medullary areas as well as where vascular congestion and necrosis were present (Fig. 2B2). Marked collagen deposition (Fig. 3B1–2) and fat tissue changes (Fig. 4B) were recorded in both the cortex and medullary regions [60]. The vasoconstriction induced by CCl₄ produced a local ischemic environment, which lead to multiple cellular damages, including the deterioration of membrane integrity [60]. In our study, the NGN + CCl₄ treated group showed almost normal morphology and normal architecture of the kidney (Figs. 2C1–2, 3C1–2 and 4C). Other reports indicated renal structural protection of naringenin against cadmium [50], whereas naringin showed nephroprotection against glycerol action in rats [61].

### 3.3. Protective effects of naringenin against TGF-β1 renal overexpression induced by CCl₄

The protective effects of NGN on the TGF-β1 expression, elevated by CCl₄ profibrotic activity, are shown in Fig. 5. The kidney slides of the control mice did not show substantial TGF-β1 immunopositivity (Fig 5A1–2). The TGF-β1 expression in the group receiving NGN alone was similar to the controls (Fig. 5D1–2). By contrast, strong TGF-β1 expression was observed for the CCl₄ group (Fig. 5B1–2). TGF-β1 immunoreactivity was observed mainly in the mesangial cells of the glomeruli and tubulointerstitial areas, showing as a predominantly brown staining. The former decreased in the kidney of CCl₄-intoxicated mice pretreated with NGN (Fig. 5C1–2).

### 3.4. Protective effects of naringenin against TIM-1 renal overexpression induced by CCl₄

TIM-1 – T-cell immunoglobulin (TIM-1) or kidney injury molecule-1 (KIM-1) is considered a renal injury biomarker [62]. In our study, TIM-1 expression was located in cortical area, slightly in the proximal tubules, for the toxic group, in accordance with other findings [63,64]. Immunopositivity was absent in the pre-treated NGN group, as well as flavonoid alone group (Fig. 6).
Electron microscopic examination of the renal cortex of the control group revealed a normal aspect of the proximal convoluted tubules, where the epithelial cells lining appeared with euchromatic nuclei and an apical long brush border, deep basolateral plasma membrane infoldings and basal vertical mitochondria (Fig. 7A1). The cells of the distal convoluted tubules exhibited euchromatic nuclei, extensive basolateral plasma membrane infoldings, short apical plasma membrane microprojection and a small population of apical cytoplasmic vesicles (Fig. 7A2).

As far as we know, no report about ultrastructural injuries in the kidneys of mice exposed to CCl₄ exists. Our ultrastructural investigation of the CCl₄-treated group revealed multiple alterations of the proximal tubular epithelial cells (Fig. 7B1–5). Furthermore, the tubules were dilated with an increase in the invagination of the dilated basal cell membrane, resulting in large irregular spaces lined by the plasma membrane (Fig. 7B2). Altered mitochondria were also observed (Fig. 7B3–4). Additionally, there was a marked increase of electron dense lysosomes-like structures with the presence of numerous lysosomes and dense granular bodies which varied in size and shape (Fig. 7B1); the latter could be dense secondary lysosomes or a mass formed by the fusion of such lysosomes [65]. Large “myeloid bodies”, indicative of an inhibited or altered function of the intralysosomal enzymatic machinery, were also present [66]. Such “myeloid bodies” were observed in renal biopsies of patients exposed to gentamicin, vio- mycin or chromium [67] as well as in kidneys of Swiss ICR mice treated with 2-bromoethylamine hydrobromide [68]. Also, myeloid bodies formation was noticed in hepatocytes after CCl₄ administration [69]. It seems that these appear as a result of the impairment of biodegradation of polar lipids associated with the decrease of lysosomal enzymes activity which cause the lysosomes enlargement and finally their breakdown with the release of myeloid bodies into the tubular lumen with concomitant tubular damage [70].

Moreover, some tubules are characterized by lipid drops accumulation (Fig. 7B5), whereas a similar effect was reported for hepatocytes following CCl₄ action [71]. The distal tubules are also affected, with epithelial lining cells presenting basal enfolding dilatations (Fig. 7B6). These results are in accordance with other reports concluding that the first segments of proximal tubules are the renal cells types most frequently involved in nephrotoxic processes, followed later by distal tubuli [66]. The group benefiting from a pre-treatment with naringenin (NGN + CCl₄) showed a marked improvement of the ultrastructural aspect of the proximal and distal tubular cells, which was comparable to that of the control group (Fig. 7C1–2).

To summarize, our results (Fig. 8) indicate an overall improvement of the structural and ultrastructural integrity of the kidney and renal antioxidant defenses upon pre-administration of naringenin to CCl₄ treated mice.

4. Conclusions

The present study suggests that the flavonoid naringenin has a renal protective potential. It effectively counteracts the CCl₄-induced oxidative damage and morphological injury at kidney level by conserving the endogenous antioxidant mechanism and scavenging free radicals. As a result, consumption of fruit or vegetables enriched in this flavonoid can protect humans against nephrotoxins and help maintain healthy kidneys. Additionally, it appears that naringenin efficiently counteracts the oxidative stress induced by several toxins.
Conflict of interest statement

The authors declare that there are no conflicts of interest.

References


