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Phloretin and phloridzin guard against cisplatin-induced nephrotoxicity in mice through inhibiting oxidative stress and inflammation

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ABSTRACT

Aim: Cisplatin (Cis) is widely used chemotherapeutic and has some serious side effects as nephrotoxicity. Phloretin (PH) and Phloridzin (PZ) are known their anti-oxidant anti-inflammatory effects. We aimed to examine the protective effects of PH and PZ on cisplatin-induced nephrotoxicity.

Main methods: Totally, 48 Balb/C female mice were separated into eight groups ($n = 6$). First day, single dose of cisplatin (20 mg/kg intraperitoneal) was administered to induce toxicity. PH and PZ were given (50 and 100 mg/kg orally) to treatment groups during 3 days. After the experimental procedures serum renal function enzymes (BUN and Creatinine), oxidative parameters (SOD, GSH and MDA), nuclear agent NFKβ, inflammatory cytokines (Tnf-α and IL1β) and HSP70 expressions and histopathological assessments were analyzed.

Key findings: Serum enzymes, tissue cytokines and oxidative stress were increased after the Cis treatment. PH and PZ treatments normalized all parameters compared to Cis administrated group. After the treatments, SOD activities and GSH levels were increased while MDA levels were decreased. PH and PZ treatments decreased Tnf-α, IL1β and NFKβ mRNA expressions. Cis significantly increased the HSP70 expression while PH and PZ administrations significantly decreased. Similar the biochemical and molecular results, PH and PZ showed positive effects on tissue pathological parameters. Cisplatin cause a lot of abnormal structures as tubular and glomeruli damages on the kidney.

Significance: PH and PZ play important physiological roles in the prevention of nephrotoxicity. Antioxidant and anti-inflammatory effects of PH and PZ demonstrated visible protective effects in the cisplatin-induced nephrotoxicity model.

1. Introduction

Cisplatin (Cis) is an organic platinum derivative and one of the well-known chemotherapeutic agents used in cancer treatment [1]. Chemotherapeutic drugs destroy rapidly growing cancer cells as well as destroy healthy cells. For this reason, most cancer drugs have some side effects on healthy cells and tissues [2]. Nephrotoxicity is one of the most common side effects of Cis treatment [3].

Cis affects the proximal and distal tubules and destroys cell membrane and increases reactive oxygen species (ROS) and as a result of this Cis treatment induces lipid peroxidation, inflammation and hypoxia [3,4]. Cis also causes ROS dependent tubular damage and renal apoptosis [5]. Renal tubular damage may lead a rise in blood urea nitrogen (BUN) level, high serum creatinine levels [3]. On the other hand some inflammatory cytokines and agents were activated by regulatory cells during nephrotoxicity [6,7]. Tumor necrosis factor alpha (Tnf-α), interleukin 1 beta (IL1β) and nuclear factor kappa beta (NFKβ) play central role in the inflammatory response triggered by Cis [6]. Heat shock protein 70 (HSP70) is released from cisplatin-injured proximal tubular cells and initiate immune response in Tnf-α dependent manner [8]. For this reason inflammatory cytokines and their second messengers are important targets for the nephrotoxicity treatments.

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There is no any exact treatment way for nephrotoxicity yet. Some chemical drugs are used against Cis induced nephrotoxicity but combined chemical drug treatment may cause more damage on kidney [9]. For this reason natural compounds can be used on the treatment because of their fewer side effects. Phloretin (PH) and its glycosylated form phloridzin (PZ) are important plant-derived dihydrochalcones which are mostly found in apple fruit and apple tree leaves [10]. Dihydrochalcones are unusual groups of natural antioxidant and the bioactivity of dihydrochalcones has attracted the attention of scientists in recent years [11]. For example, a typical dihydrocolic phloretin has been investigated for its anti-inflammatory and hepatoprotective effects in mouse models [12,13]. LoPachin et al. showed phloretin has a strong oxidant scavenging effects and can provide cytoprotection in cell culture and animal models of oxidative stress injury [14]. It has also been suggested that glucoside phloridzin (phloretin 2′-β-d-glucoside) has cytoprotective effects [15]. Phloretin and phloridzin, major flavonoid structured compounds of apple, is well known to play important role on glucose absorption [16]. Phloridzin blocks intestinal glucose absorption and increases renal glycosuria through inhibition of the sodium–glucose co-transporters located in the proximal tubule of kidney and mucosa of the small intestine [17]. Phloretin, an inhibitor of glucose transporter 2, also causes glucose uptake inhibition and regulates ion channels and exert beneficial roles upon kinds of cell types [18]. In addition to their protective effect of glucose metabolism they have proven to be stronger antioxidants than the well-known flavonoids due to the presence of the two hydroxyl (-OH) groups in their chemical structure [19]. It is well known that -OH groups can promote the antioxidant activities of flavonoids [20]. Also anti-proliferative effects of phloretin against cancer cells have been reported, supporting their combination with cisplatin to both decrease its side effects and potentiate its anticancer effects [21].

In the present study, we aimed to examine the potential therapeutic effects of phloretin and phloridzin on the basis of mentioned biological activations of these compounds against cisplatin nephrotoxicity in mice.

2. Material and methods

2.1. Animals

Totally, 48 Balb/C female mice were used in this experiment. Each mouse weighed 30–40 g and was obtained from Ataturk University Experimental Animal Laboratory. The Ethics Committee of Ataturk University approved the study, with protocol number 2019-4/71.

2.2. Chemicals

Cisplatin (Kocak Farma, Turkey), Phloretin (CAS: 60-82-2), phloridzin (CAS: 7061-54-3) and all chemicals that we used during the experimental process were purchased from Sigma Chemical (Germany).

2.3. Cisplatin-induced nephrotoxicity model

Totally 48 mice were separated into eight groups ($n = 6$) in line with previous studies [22,23]. First day, single dose of cisplatin (20 mg/kg intraperitoneal) was administered to induce toxicity [24]. Phloretin and phloridzin were given (50 and 100 mg/kg orally) to treatment groups during 3 days. Doses were selected in accordance to previous toxicity and efficacy studies performed in laboratory animals [25,26]. The experimental design and groups were shown in Table 1. All mice were killed with an overdose general anesthetic (thiopental sodium, 50 mg/kg). Blood samples were collected and kidneys were dissected for biochemical, histopathological and molecular analyses. Blood samples were collected via the intracardiac method. The serum was separated by centrifugation at 4000 rpm for 10 min at 4 °C and stored at −80 °C. The kidney tissues were dissected out and half of one kidney was kept at −80 °C for biochemical analysis, the other half of the kidney was kept in RNA stabilization reagent for molecular analysis. Second kidney was fixed in a 10% formalin solution for histopathological investigation.

Table 1
Drug administrations and experimental procedures.

Groups	1 h before Cis treatment	Cis treatment	24 h after Cis treatment	48 h after Cis treatment	72 h after Cis treatment
Healthy	Water	Water	Water	Water	Sacrified
Healthy + PH2	100 mg/kg PH	water	100 mg/kg PH	100 mg/kg PH	Sacrified
Healthy + PZ2	100 mg/kg PZ	water	100 mg/kg PZ	100 mg/kg PZ	Sacrified
Cis	Water	20 mg/kg Cis	Water	Water	Sacrified
Cis + PH1	50 mg/kg PH	20 mg/kg Cis	50 mg/kg PH	50 mg/kg PH	Sacrified
Cis + PH2	100 mg/kg PH	20 mg/kg Cis	100 mg/kg PH	100 mg/kg PH	Sacrified
Cis + PZ1	50 mg/kg PZ	20 mg/kg Cis	50 mg/kg PZ	50 mg/kg PZ	Sacrified
Cis + PZ2	100 mg/kg PZ	20 mg/kg Cis	100 mg/kg PZ	100 mg/kg PZ	Sacrified

Healthy: Nontreated group, Cis: Cisplatin, PH: Phloretin, PZ: Phloridzin.

2.4. Biochemical investigation

After the surgical procedures, kidney tissues were fixed with liquid nitrogen. Subsequently 50 mg of pulverized tissue samples were mixed with 1 ml PBS and ground in liquid nitrogen using a Tissue Lyser II (Qiagen) grinding set. All samples were centrifuged and supernatants were used as sample. Superoxide dismutase (SOD) activity [27], glutathione (GSH) [28] and malondialdehyde (MDA) [29] levels were measured in duplicate according to the modified methods with multi well plate reader [30]. Data were presented as the mean ± standard deviation results per mg of protein. Total protein levels were analyzed by the Lowry Method, using commercial protein standards (Sigma Aldrich, TP0300).

2.5. Renal function tests

Blood samples were centrifuged and serums were collected. Commercial kits were used to analyze renal function tests (BEN Biochemical Enterprise). BUN (Ref: BK151) and Creatinine (Ref: Cr280) levels were measured with multi well plate reader.

2.6. Molecular investigations

2.6.1. Total RNA extraction and cDNA synthesis

Kidney tissues (20 mg) were stabilized in an RNA stabilization reagent (RNAlater, Qiagen) and then shredded using the Tissue Lyser II (Qiagen).Total RNA was purified using RNeasy Mini Kit Qiagen according to the instructions of the manufacturer in QIAqube (Qiagen). The RNA samples were reverse-transcribed into complementary DNA using a high-capacity cDNA reverse transcription kit (Applied Biosystem). The cDNA concentrations were assessed and quantified using the Epoch Spectrophotometer System and Take3 Plate (Biotek) [31].

2.6.2. Relative quantification of gene expression

Relative Tnf-α, IL1β, NFKβ and HSP70 expression analyses were performed with StepOne Plus Real Time PCR System technology (Applied Biosystem). Quantitative PCR was run by using TaqMan Probe mix (Applied Biosystem). Real-time PCR was performed using primers generated for all genes. The expression data for β-actin in each tissue were used as endogenous controls. For each tissue, triplicate determinations were performed in a 96-well optical PCR plate and all quantification of gene expression steps were performed as described previously [32]. All data were expressed as fold changes in expression compared to expression in other animal groups using the 2-ΔΔCt

method [33].

2.7. Histological investigations

Kidneys from the mice were taken at necropsy and fixed in 10% neutral formalin solution. Then, the tissues were processed routinely and tissues were embedded in paraffin wax, and 4–5 μm thick serial sections were cut. All tissue sections were stained with hematoxylin and eosin for histopathology assessment before being examined under a light microscope (Olympus BX51, Japan). For histopathological assessment, corticomedullary tubular damage rate was evaluated for kidney tissues. A minimum of five fields for each kidney slide at x200 magnification were evaluated and assigned severity of change scores according to the following scales: Grade 1: 1–10%, Grade 2: 11–25%, Grade 3: 26–50%, Grade 4: 51–75%, Grade 5: 76–100% tubular damage, as described previously [34].

2.8. Statistical analysis

SPSS 25.0 software was used for all the statistical analysis and the results were presented as means ± standard deviation (S.D.). Statistical analysis of gene expressions and biochemical results were performed using one-way ANOVA and the Tukey multiple comparison test. Significant differences were detected between all groups, compared to Healthy group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$), compared to Cis group (*p < 0.05, **p < 0.01, ***p < 0.001) and compared to Cis + PZ2 group ($^{\delta}$p < 0.05, $^{\delta\delta}$p < 0.01, $^{\delta\delta\delta}p < 0.001$).

3. Results

3.1. Biochemical results

SOD, GSH and MDA levels were shown in Fig. 1. Cis administration significantly increased MDA level and significantly decreased SOD activity and GSH level when compared to Healthy group (p < 0.001). Looking at the Fig. 1, it was shown that both doses of phloretin and phloridzin ameliorated SOD activities as well as GSH and MDA levels in the Cis + PH and Cis + PZ groups. High doses of phloretin and phloridzin significantly increased SOD activities and GSH levels while decreased MDA levels compared to Cis group ($p < 0.001$). Furthermore, there were no significant differences in baseline values of SOD, GSH and MDA between the Healthy and high doses of PH and PZ treated Healthy groups.

3.2. Renal function test results

Serum BUN and Creatinine (Fig. 2) concentrations were significantly increased by Cis administration compared to the Healthy group ($p < 0.001$). It was shown that PH and PZ treatments ameliorated renal function parameters. High dose of PH (100 mg/kg) significantly decreased BUN ($p < 0.001$) and Creatinine ($p < 0.01$) levels when compared with Cis group. As shown in Fig. 2, 100 mg/kg of PZ administration significantly decreased the BUN and Creatinine levels (p < 0.001). However PH and PZ treatments did not changed these levels in healthy groups.

3.3. Gene expression results

Tnf-α expression increased in Cis group by 8.2-fold compared to healthy group ($p < 0.001$) (Fig. 3). PH and PZ administrations decreased Tnf-α expressions to 4.2-fold in Cis + PH1, 3.1-fold in Cis + PH2, 7.6-fold in Cis + PZ1 and 6.7-fold in Cis + PZ2 group compared to Cis group. It was shown that Tnf-α expression was significantly decreased in Cis + PH2 group compared to Cis + PZ2 group ($p < 0.001$).

Similarly IL1β gene expression also increased in Cis group by 20.4-fold compared to Healthy group ($p < 0.001$) (Fig. 3). Both doses of PH and PZ significantly decreased IL1β gene expression when compared

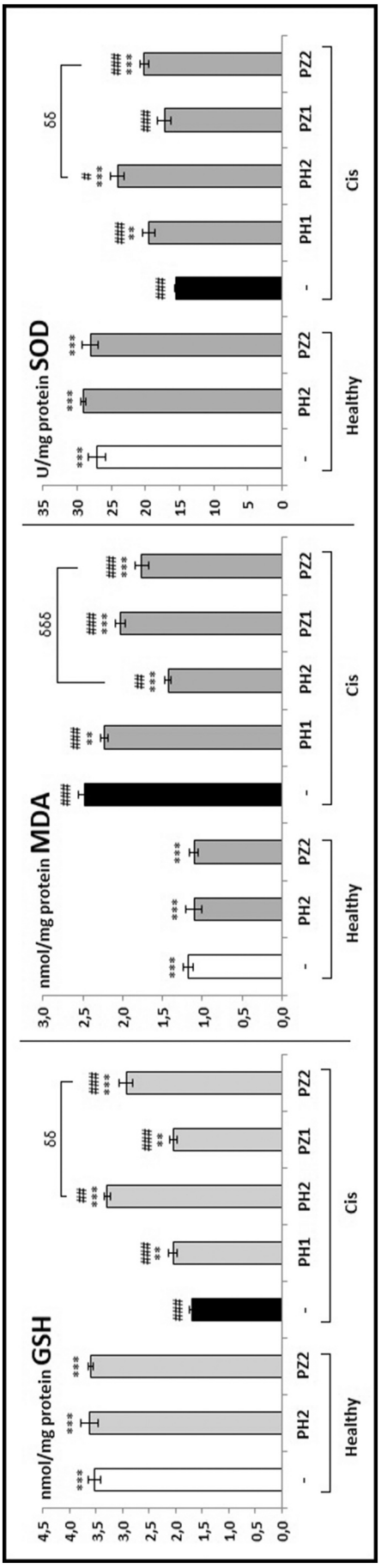


Fig. 1. Biochemical results of PH and PZ treatments in the kidney tissues. Cis: Cisplatin (20 mg/kg i.p.), PH1: 50 mg/kg phloretin, PH2: 100 mg/kg phloretin, PZ1: 50 mg/kg phloridzin, PZ2: 100 mg/kg phloridzin treatments. GSH: Total glutathione levels, MDA: Malondialdehyde levels, SOD: Superoxide dismutase activities. Each bar expressed as mean value ± S.D. Significant differences were detected between all groups, compared to Healthy group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$), compared to Cis group (**p < 0.01, ***p < 0.001) and compared to Cis + PZ2 group ($^{\delta\delta}$p < 0.01, $^{\delta\delta\delta}$p<0.001) by one-way ANOVA followed by Tukey test.

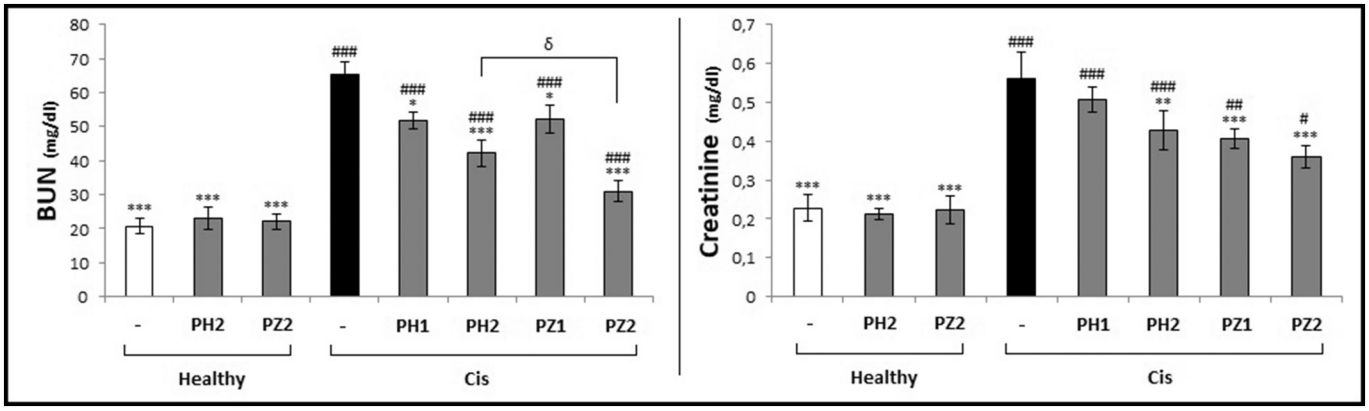


Fig. 2. Serum BUN and Creatinine levels. Cis: Cisplatin (20 mg/kg i.p.), PH1: 50 mg/kg phloretin, PH2: 100 mg/kg phloretin, PZ1: 50 mg/kg phloridzin, PZ2: 100 mg/kg phloridzin treatments. Each bar expressed as mean value ± S.D. Significant differences were detected between all groups, compared to Healthy group ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$), compared to Cis group ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) and compared to Cis + PZ2 group ($^{\delta}p < 0.05$) by one-way ANOVA followed by Tukey test.

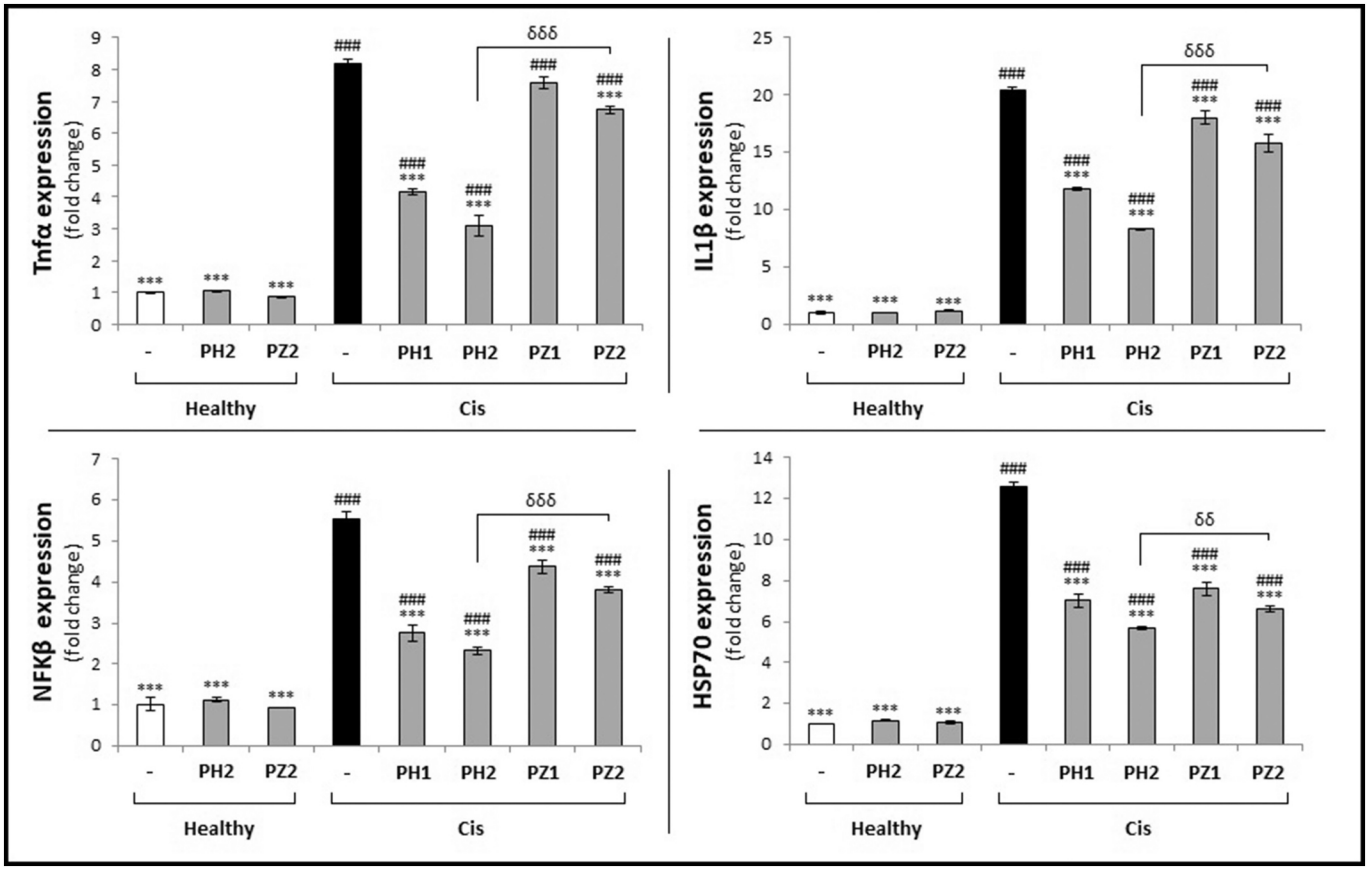


Fig. 3. Relative mRNA expression levels of Tnfα, IL1β, NFKβ and HSP70 in the kidney tissues. Cis: Cisplatin (20 mg/kg i.p.), PH1: 50 mg/kg phloretin, PH2: 100 mg/kg phloretin, PZ1: 50 mg/kg phloridzin, PZ2: 100 mg/kg phloridzin treatments. The expression of mRNAs was detected using quantitative Real-Time PCR analysis. β-Actin was used as the reference gene. Results are expressed as relative fold compared with healthy animals. Each bar expressed as mean value ± S.D. Significant differences were detected between all groups, compared to Healthy group ($^{\#\#\#}p < 0.001$), compared to Cis group ($^{***}p < 0.001$) and compared to Cis + PZ2 group ($^{\delta\delta}p < 0.01$, $^{\delta\delta\delta}p < 0.001$) by one-way ANOVA followed by Tukey test.

with Cis group ($p < 0.001$). IL1β gene expressions were analyzed in Cis + PZ2 group by 15.7-fold and Cis + PH2 group by 8.28-fold.

Looking at the Fig. 3, it was shown that Cis administration significantly increased NFKβ expression by 5.5-fold compared to Healthy group ($p < 0.001$). Both doses of PH and PZ significantly decreased NFKβ gene expression when comparing with Cis group ($p < 0.001$). NFKβ gene expressions were analyzed in Cis + PZ2 group by 3.8-fold and Cis + PH2 group by 2.3-fold.

HSP70 mRNA expression increased in Cis administrated group by 12.6-fold compared to Healthy group ($p < 0.001$) (Fig. 3). PH and PZ administrations decreased HSP70 expressions to 7.1-fold in Cis + PH1, 5.7-fold in Cis + PH2, 7.6-fold in Cis + PZ1 and 6.5-fold in Cis + PZ2 group compared to Cis group. It was shown that HSP70 expression was significantly decreased in Cis + PH2 group compared to Cis + PZ2 group

($p < 0.01$).

3.4. Histopathological results

Histopathological changes in the kidneys were examined for phloretin on Fig. 4 and phloridzin on Fig. 5. In Healthy group, the general appearance and borders of the cortex and medulla were seen as normal. It was also shown that renal corpuscles, proximal and distal tubules located in the cortical region and collecting tubules located in the medulla had a healthy appearance in healthy group (Fig. 4A). The appearance of the Healthy+PH2 group was similar to the Healthy group. No abnormality was observed due to high dose phloretin treatment (Fig. 4B). Looking at the Fig. 4C, it was shown that intense leukocyte infiltration was observed in the arcuate artery and arcuate vein line (arrow). It has also been determined that some glomeruli had smaller diameters and the Bowman interval was wider than normal. The shrinking glomeruli were found to have an intense eosinophilic appearance (curved arrow). It was analyzed that the wall integrity of the proximal tubules disappeared due to damage, mainly in the sub capsular region (arrowhead) (Fig. 4C). In the Cis + PH1 group, lymphocyte infiltration and inflammation findings were shown on the corticomedullary border when compared to the Cis group (arrow) as shown in Fig. 4D. Bowman range has shown to be normal size even though some glomeruli shrink in diameter (curved arrow). The appearance of proximal and distal tubules was normal. Increased tubular epithelial cells were observed (Fig. 4D). Looking at the Healthy+PH2 group in Fig. 4E, it was revealed that fewer leukocytes were found around the arcuate artery when compared to Healthy+PH1 group. The general view of the section was similar with the Healthy group.

Looking at the healthy group in Fig. 5A, abnormalities were not detected in the general view of the cortical and medullar areas. Proximal and distal tubules and glomeruli structures were seen as healthy (Fig. 5A). No histopathological findings were observed in high dose phloridzin treatment and the view of the section was similar to the Healthy group in the Healthy+PZ2 group (Fig. 5B). In the Cis administered group (Fig. 5C), severe leukocyte accumulations were observed on the corticomedullary line (arrow). Some disappeared glomeruli and accumulated cell debris was observed (arrowhead). In some folded proximal tubules, dense eosinophilic staining has been detected which is possibly associated with necrotic damage (thick arrow). In the sub capsular region, tubular damages (vaculations and desquamization) were more intense than in other regions (thick arrow) (Fig. 5C). In the Cis + PH1 group inflammation was shown around the lobular artery and veins (arrow) (Fig. 5D). In the medullary region, dense eosinophilic stained thin tubules (thin limbs) were observed due to possible damage (curved arrow). It was also shown that there were signs of edema in the

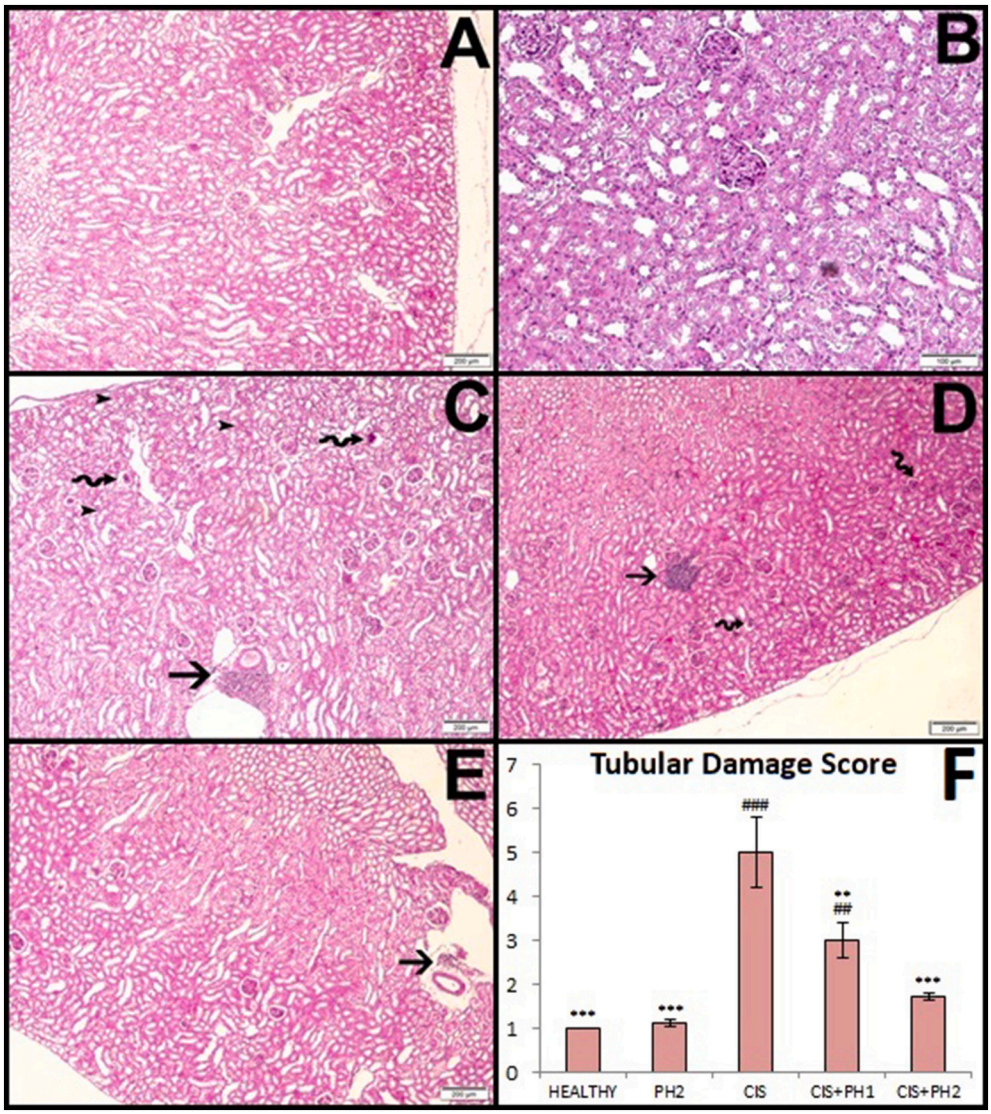


Fig. 4. Heamatoxylin and eosin staining and histological evaluation results of PH on the Cis-induced nephrotoxicity; Magnification 200×. Cis: Cisplatin (20 mg/kg i.p.), PH1: 50 mg/kg phloretin, PH2: 100 mg/kg phloretin treatments. A (HEALTHY) and B (HEALTHY + PH2): Normal structures of the kidney. C (Cis): Leukocyte infiltration (arrow), intense eosinophilic appearance (curved arrow) and damaged proximal tubules (arrowhead) are seen. D (Cis + PH1): Some lymphocyte infiltration (arrow) and increased tubular epithelial cells are seen. E (Cis + PH2): Normal kidney structure is seen. F: Damaged tubular score results are seen. Grade 1: 1–10%, Grade 2: 11–25%, Grade 3: 26–50%, Grade 4: 51–75%, Grade 5: 76–100% tubular damage. Results are expressed as relative fold compared with healthy animals. Each bar expressed as mean value ± S.D. Significant differences were detected between all groups, compared to Healthy group ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$), compared to Cis group ($^{**}p < 0.01$, $^{***}p < 0.001$) by one-way ANOVA followed by Tukey test.

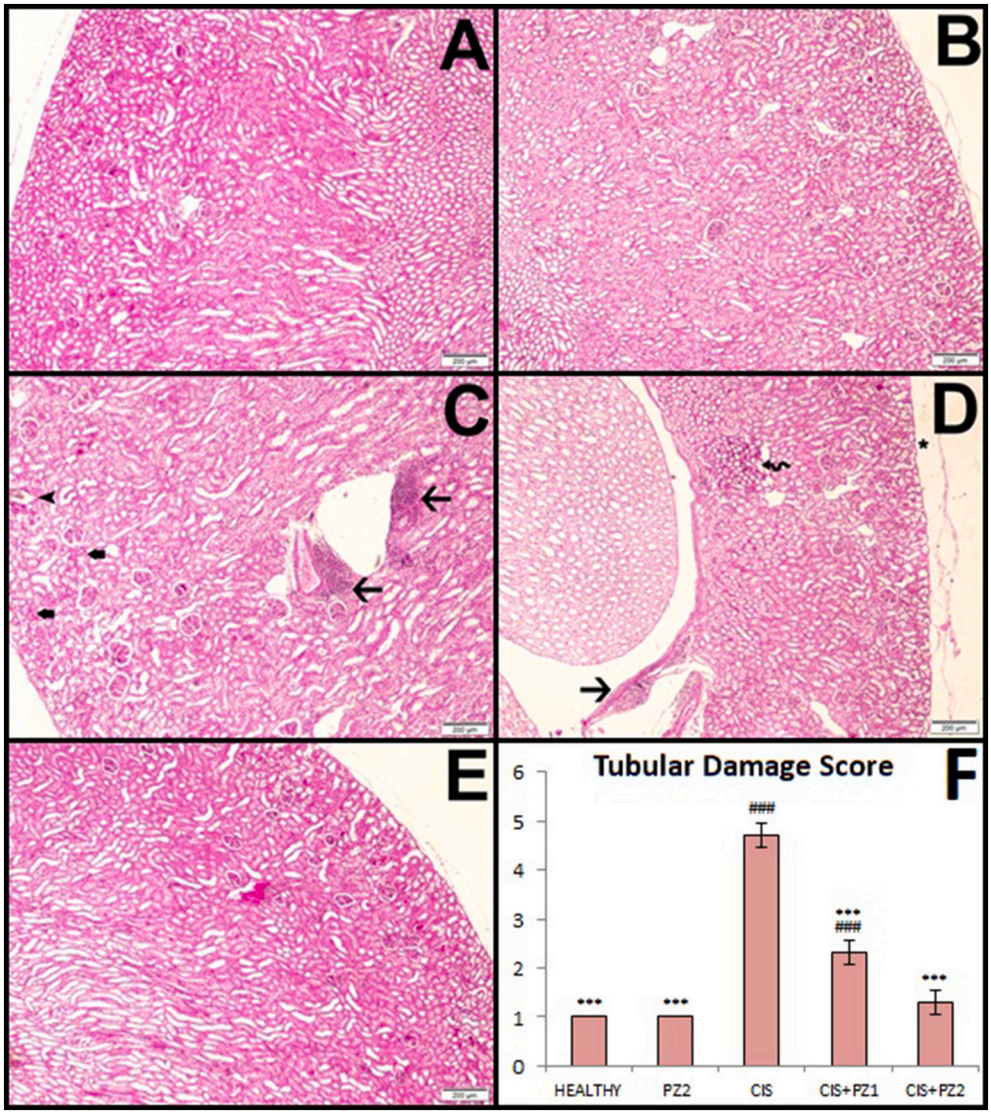


Fig. 5. Heamatoxylin and eosin staining and histological evaluation results of PZ on the Cis-induced nephrotoxicity; Magnification 200×. Cis: Cisplatin (20 mg/kg i.p.), PZ1: 50 mg/kg phloridzin, PZ2: 100 mg/kg phloridzin treatments. A (HEALTHY) and B (HEALTHY + PZ2): Normal structures of the kidney. C (Cis): Leukocyte accumulations (arrow), cell debris (arrowhead) and dense eosinophilic staining and tubular damages (thick arrow) are seen. D (Cis + PZ1): Signs of edema (star), less inflammation (arrow) and eosinophilic stained thin tubules (curved arrow) are seen. E (Cis + PZ2): Normal kidney structure is seen. F: Damaged tubular score results are seen. Grade 1: 1–10%, Grade 2: 11–25%, Grade 3: 26–50%, Grade 4: 51–75%, Grade 5: 76–100% tubular damage. Results are expressed as relative fold compared with healthy animals. Each bar expressed as mean value ± S.D. Significant differences were detected between all groups, compared to Healthy group ($^{###}p < 0.001$), compared to Cis group (***$p < 0.001$) by one-way ANOVA followed by Tukey test.

sub capsular area (star) in Cis + PH1 group (Fig. 5D). Looking at the high dose phloridzin administered group (Cis + PZ2), the general view of the cortical and medullar regions were similar to the Healthy group. Edema areas in the sub capsular region and damage findings in the tubular region were significantly reduced compared to the Cis + PZ1 group (Fig. 5E).

4. Discussion

In this study we examined the potential therapeutic effects of PH and PZ in cisplatin-induced nephrotoxicity in mice. Phloretin and phloridzin ameliorated the oxidative tissue damage, inflammation response against to nephrotoxicity damage and renal functions.

Nephrotoxicity is a complex disease which causes both activation of oxidative stress and inflammation [9]. The main mechanism of toxicity is tubular cells damage by chemical agent. Renal tubular cell membrane is damaged by this chemical agent and causes the releasing the ROS. Increased levels of ROS first interact with glutathione which is one the most important anti-oxidant molecule on protecting cells from oxidative stress [5,35]. Second, interact the mitochondrial respiratory chain to produce more ROS [35]. There is a direct relation between the anti-oxidant treatment and decreased ROS level. Anti-oxidant supplementations have been considered for treatment of nephrotoxicity as well [35]. Kinds of natural anti-oxidant molecules have been evaluated to be helpful in reducing cisplatin-induced nephrotoxicity [36,37]. A research team examined in their mice studies that excessive level of oxidants amplifies a marker for nephrotoxicity which is promptly reversed normal levels with natural flavonoid supplementations [38].

PH and PZ are the kinds of dihydrochalcones that are mostly known their antioxidant effects like the all other subfamily members of flavonoids [19]. The antioxidant capacities of different dihydrochalcone compounds have been shown in the literature [39]. These compounds show radical scavenging effects via their phenolic -OH groups. It has been examined that phenolic hydroxyl groups included compounds are considered to be excellent antioxidants that can quench excessive free radical-induced tissue damage and chronic diseases [18,40]. It has been also shown that one of the important antioxidant ability center of flavonoids is phenolic hydroxyl, so the number and position of phenolic hydroxyls are directly related with their antioxidant activity [20]. PH and PZ can cause more radical scavenging effects than other flavonoids in oxidative damage because of theirs different positioned and more hydroxyl groups [19].

In order to prevent oxidative damage, antioxidants play an important role in protecting against cisplatin-induced nephrotoxicity [36–38]. GSH is one of the most effective antioxidant in living organisms which is important in protecting against to renal injury. Moreover it scavenges

reactive oxygen species in nephrotoxicity [41]. In the present study, the GSH content in the Cis group was substantially decreased. Phloretin and phloridzin treatments exhibited significant increases in GSH content. SOD is an antioxidant enzyme that eliminates ROS. Reduction in SOD activity in cisplatin exposed animals may be due to the overproduction of superoxide radical anions [35]. In the present study, SOD enzyme activity in the Cis group was significantly lower compared to healthy group. However, the kidney SOD enzyme activity in both doses of the PH and PZ treated groups significantly increased when compared with the cisplatin group.

As another result of oxidative damage in nephrotoxicity, ROS cause a separation of hydrogen atoms from unsaturated lipid and initiating lipid peroxidation [29]. Lipid peroxidation results in several changes in membrane structure and functions of the cell and cause DNA damage, cytotoxicity, and eventually cell death. MDA is the end product of lipid peroxidation and often used as a biomarker to measure the level of oxidative stress in organisms [42]. In the present study, the MDA level has dramatically increased in the cisplatin treated group comparing with the healthy group. This situation is closely related to cisplatin-induced lipid peroxidation and damage to plasma membrane because of oxidative stress. However, phloretin and phloridzin administrations significantly decreased MDA levels. This effect might be due to the potential antioxidant activities of PH and PZ. Previous studies have also showed the antioxidant effects of PH and PZ against to cell toxicity [11]. Supporting to our results in another toxicity study, researcher have showed PH and PZ normalized aforementioned antioxidant mechanisms [15].

Together with oxidative stress, inflammation is another important factor in cisplatin-induced nephrotoxicity. Cisplatin has been found that plays an inflammatory role in kidney [43]. It is well-known that cisplatin causes phosphorylation and translocation of NFKβ from cytoplasm into nucleus. Activated NFKβ promotes transcription of Tnf-α and IL1β [44]. Subsequently immune cells contribute to the development of renal inflammation resulting in enhanced production of inflammatory cytokines [45]. Inhibition of these cytokines may be useful on the nephrotoxicity treatment. It was shown that natural flavonoids inhibited the inflammatory cytokines in cisplatin-induced nephrotoxicity [38]. Similarly to this study we showed that PH and PZ ameliorated the excessive levels of NFKβ and inflammatory cytokines Tnf-α and IL1β levels. In another study in the literature it was examined that phloridzin suppressed the NFKβ translocation to nucleus and decreased inflammatory cytokine levels [18].

In response to oxidative stress and inflammation, renal tubular cells may activate multiple cyto-protective mechanisms for cell survival [46]. The heat shock response is one of the cellular mechanisms of protection under cell stress. Under the stress condition induced by chemical toxins HSP70 proteins appear to protect vital proteins from denaturation [46]. HSP response has been reported in experimental kidney injury model [47]. It was also showed that HSPs play important role on cisplatin-induced nephrotoxicity [48]. HSP is originally described as an intracellular chaperone, but it has been also reported that HSP had anti-inflammatory effects as well [49]. In our study we showed that Cis induced nephrotoxicity caused an increase of HSP70 expression similar to previous studies. However, interestingly PH and PZ treatments decreased HSP70 levels unlike Tnf-α and IL1β expression levels. Probably PH and PZ treatment first decreased inflammatory cytokines and then decreased cytokines suppressed the HSP70 levels. It has been also showed that there were positive correlations between HSP70 levels and Tnf-α and IL1β levels in inflammatory condition [50]. In our study we examined that PH and PZ administrations decreased all these gene expressions compared to cisplatin treated group. Controversially to our results it was declared that Phloretin increased the HSP70 activation in tumor cells [51]. Generally most of natural compounds show controversially effects on cancer cells and normal cells. Cancerous cells is activated their selves uncontrolled manner and produce excessive amount of protein. In response to these activations some natural agents act different against to huge amount of cancer cells [52]. In another cancer research it was shown that both PH and PZ decreased the cell proliferation [53]. Natural compounds that have anti-cancer effects can protect healthy cells while they defect the cancerous cells. For this reason these compounds may be used in combined with chemotherapeutics to decrease its side effects and potentiate its anticancer effects [21].

The main diagnostic parameter of nephrotoxicity is serum BUN and Creatinine levels. The concentration of BUN reflects glomerular filtration and urine-concentrating capacity [54]. The increased tubular secretion of creatinine related to kidney dysfunction [54]. It was shown that cisplatin treatment caused an increase of BUN and creatinine levels [36]. Similar to these results we examined increased levels of BUN and creatinine in cisplatin-induced nephrotoxicity. There are a lot of researches in the literature which are related with natural compound's suppressive effects of on these renal function parameters. It was shown that apigenin and myricetin, which are natural flavonoids, decreased BUN and creatinine levels compared to cisplatin administration [38]. In our study we showed the similar effects of PH and PZ against to cisplatin induced nephrotoxicity. It was previously showed that phloretin ameliorates hyperuricemia-induced chronic renal dysfunction by decreasing serum BUN levels [55]. Similar to our results phloridzin have been decreased BUN and creatinine levels in diabetic nephropathy [56]. Recent studies support our results and show the potential protective effects of PH and PZ via its antioxidant and anti-inflammatory effects.

Previous studies also reported many different mechanisms such as regulation of glucose metabolism [16], inhibition of sodium glucose transporters [17], inhibition of several ion channels [18] etc. as contributing factors to pharmacodynamics effects of PH and PZ. Beyond the mechanism we discussed above, additional studies are required to investigate possible relationship between all these mechanisms and nephroprotective effects of PH and PZ.

Finally looking at the histopathological data, the results supported the molecular and biochemical findings. The kidney histology of the mice was impaired after the Cis administration and started to improve following PH and PZ administrations. It has well known that Cis administration caused cellular and tubular damage [57]. Phloretin and phloridzin treatments showed improvement of the tubular degenerations in dose dependent manner. This improvement could be related with kidney function parameters BUN and Creatinine because PH and PZ administrations ameliorated their levels. High dose PH and PZ treatments, after the Cis administration, normalized the kidney's histo-architecture. After the treatment it has revealed that phloretin and phloridzin have positive effects on histopathological changes especially on tubular degeneration, vacuolization and collapse glomerulus.

This study has some limitations to be addressed. First; this study was performed on healthy animals so we cannot directly conclude about the positive interaction of PZ and PH with cisplatin during cancer treatment, but we suggest that they can prevent kidney tissue from cisplatin's harmful effects. Second; for evaluating kidney injury we used serum biomarker such as BUN and Creatinine. Also urine biomarkers could be helpful but because of limited urine amount that can be collected from mice; we could not perform urine analyses. Finally, PH and PZ can be exerted protective effects via changing pharmacokinetic profile of cisplatin (changing its distribution, metabolism or elimination) and observed pharmacodynamics effects of PH and PZ can be a result of pharmacokinetic interactions. In this study we did not evaluate the pharmacokinetic profiles of PZ and PH. However, future additional analyses are required to test this possibility.

5. Conclusion

As conclusion, it was examined that phloretin and its glycosylated form phloridzin ameliorate the toxic effects of cisplatin. PH and PZ showed beneficial effects on kidney tissues by preventing releases of proinflammatory cytokines such as Tnf-α, IL1β, NF-κB, and also decreasing oxidative stress, which are markers of damage. Moreover

because of their previously reported anti-proliferative effects combining PH and PZ with cisplatin, may decrease cisplatin's side effects and potentiate its anticancer effects. This study shed light to future clinical studies that will evaluate PH and PZ as potential therapeutic candidates to reduce nephrotoxicity.

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Declaration of competing interest

The authors declared no conflicts of interest.

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