Falcarindiol attenuates cisplatin-induced nephrotoxicity through the modulation of NF-kB and Nrf2 signaling pathways in mice

Mojtaba Dolatpanah[1,2], Mehran Mesgari Abbasi[3], Saeed Nazari Soltan Ahmad[1], Jamal Mohammadian[2,3], Leila Roshangar[4], Hassan Argani[1], Nadereh Rashtchizadeh [1]*, Amir Ghorbanihaghjo[1]*

[1]Biotechnology Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[2]Department of Clinical Biochemistry and Laboratory Sciences, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran

[3] Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

[4] Stem Cell Research Center, Tabriz University of Medical Sciences, Tabriz, Iran

*Corresponding authors: Amir Ghorbanihaghjo, Nadereh Rashtchizadeh

E-mail address: **NaderehRashtchizadeh44@outlook.com**

Abstract

Cisplatin is a therapeutic drug widely used to treat various solid tumors. Nephrotoxicity is a well-known side effect in patients treated with cisplatin. Falcarindiol (FAD), natural polyacetylene compound greatly found in Apiaceae family, has anti-cancer, -bacterial, -inflammatory and -oxidant activity which is utilized in the present study. Thirty male C57BL/6 mice were randomly divided into five groups of six each; sham, cisplatin (15 mg/kg), cisplatin+FAD (50 and 100 mg/kg/day), and FAD (100 mg/kg/day). Cisplatin administration elevated the concentrations of BUN and creatinine, as well as kidney histopathologic damage. On the other hand, FAD treatment attenuated cisplatin-induced injury, and also down-regulated mRNA levels of TNF-α and IL-1β together with protein expression of p-NF-kB p65. Moreover, FAD induced the protein expression of p-AMPK and nuclear Nrf2 accompanied by its respective target genes such as NQO-1 and HO-1 in a dose-dependent manner. In conclusion, the findings collectively characterize FAD as a drug candidate to treat cisplatin-induced nephrotoxicity thorough down-regulation of NF-kB signaling pathway in mice.

Keywords: Cisplatin; Nephrotoxicity; Falcarindiol; NF-kappa B; Nrf2

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1. Introduction

Cisplatin is a platinum-containing chemotropic drug which is extensively used to treat various kinds of solid tumors [1, 2]. Cisplatin mainly reacts with reactive N7 atom on purines to form Cross-linking with DNA, and thereby blocks its replication and transcription [3]. In spite of its very potent antitumor effects, application of cisplatin is limited in clinical condition because of drug resistance as well as its toxicity in normal tissues [4-6]. Among around the forty known side effects of cisplatin, nephrotoxicity is considered one of the major complications. Nephrotoxicity is observed in 20 to 35% of cancer-affected patients who take this drug. In addition, cisplatin-induced nephrotoxicity (CIN) may lead to acute kidney injury (AKI) and mortality [7, 8]. Different mechanisms are involved in the CIN pathology which include DNA adducts formation, oxidative stress, inflammation response, disrupted mitochondria function and apoptosis [9]. Although the effectiveness of various treatment strategies such as hydration and treatment with diuretics have clinically been evaluated to prevent or improve CIN, no effective treatment presently is available to reduce its risk in the cisplatin-treated patients [8] and the nephrotoxicity is still observed in significant part of cisplatin-treated patients who have only received one dose of cisplatin [2].

Recent evidences reveal oxidative stress and inflammation play an important role in the pathophysiology of CIN [10]. Cisplatin leads to upregulation of tumor necrosis factor-α (TNF-α) and nuclear factor-κB (NF-κB) which is followed by the production of pro-inflammatory cytokines and chemokines. These events eventually cause inflammatory cells to infiltrate into kidney and exacerbate cisplatin-induced kidney injury. Therefore, evaluation of anti-inflammatory agents effectiveness in preventing or improving of CIN may promise new therapeutic approaches to treat cancer-affected patients [11].

Falcarindiol (FAD) as a natural polyyne is widely found within the members of Apiaceae family, and it has been characterized by antioxidant, anti-inflammatory, antibacterial and anticancer properties [12-14]. FAD induces gene expression of antioxidant enzymes through activation of the Kelch-like ECH-associated protein 1 (Keap1)/nuclear factor-erythroid 2–related factor 2 (Nrf2)/antioxidant response element (ARE) pathway [15]. In addition, FAD also reduces NF-κB and TNF-α expression that regulates inflammation and immune responses [15].

According to the role of oxidative stress and inflammation in CIN as well as antioxidant and anti-inflammatory effects of Falcarindiol, protective effect of Falcarindiol on cisplatin-induced nephrotoxicity is evaluated in the present study.

2. Material and methods

2.1. Animals

Present study was approved by the animal ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.VCR.REC.1400.139). Thirty five-week-old C57/BL6 mice (18-22 g) were obtained from Pasteur institute (Iran, Tehran). The standard protocols of experiments and animals care were carried out according to the approved guidelines of the Research University Council. The mice were kept in the laboratory to adapt to the environment for one week. Temperature (25±1 °C), humidity (65±1) and 12-hr dark/light cycle were controlled and Standard chow diet and water were accessible in animal cage ad libitum during the experimental phase of the study.

2.2. Study design

The mice were randomly divided into five groups (Six per group):

1) Sham group (received vehicle of cisplatin (CIS) and FAD)

2) FAD group (received Falcarindiol 100 mg/kg) [16]

3) CIS group (received cisplatin 15 mg/kg)

4) FAD 50+CIS (received Falcarindiol 50 mg/kg and cisplatin 15 mg/kg)

5) FAD 100+CIS (received Falcarindiol 100 mg/kg and cisplatin 15 mg/kg)

Falcarindiol (Selleckchem, USA) intraperitoneal injection was administered to the mice of FAD (100 mg/kg) and FAD 50+CIS (50 mg/kg) and FAD 100+CIS (100 mg/kg) groups for four consecutive days. Twelve hours after the first Falcarindiol injection, 2 ml/kg saline-0.05% dimethyl sulfoxide (DMSO)-dissolved cisplatin (Sigma, USA) (15 mg/kg) was intraperitoneally injected to the mice of the CIS and FAD+CIS groups at the single dose. The Sham group received intraperitoneally saline-0.05% DMSO at the same volume with the other groups.

Twelve hours after the last injection, the mice were anesthetized by intraperitoneal injection of ketamine-xylazine (75+10 mg/kg) and underwent surgery. Blood samples were taken from inferior vena cava, and kidneys were removed. After washing with cold and sterile PBS, a kidney of each mouse was aliquoted into some cryotubes and frizzed in liquid nitrogen. The kidney tissues were saved at -80 °C. The other kidney of each mouse was cut into proper section and fixed in formalin 10% for histopathological evaluation. The blood samples were centrifuged (3000 rpm, 10 min) to prepare sera samples.

2.3. Measurement of biochemical parameters

Serum level of blood urea nitrogen (BUN) and serum creatinine (Scr) were measured by commercial kits (Pars Azmoon, Iran).

2.4. Histopathological evaluation

Formalin-fixed kidney tissues were embedded in paraffin. Paraffin-embedded blocks were cut into five µm slides using a microtome (Leica, Germany) and the slides were fixed on glass slides. Finally, the tissue slides were stained with Hematoxylin and Eosin (H&E) method and the type and severity of tissue damage were evaluated by a pathologist in a blind manner.

2.5. Real-time Quantitative PCR assay

At first, Total RNA samples were extracted by RNAzol® RT (Sigma, USA) based on manufacturer's instructions. The quantity and quality of extracted RNA samples were evaluated by nanodrop (NanoDrop Technologies, USA) and 1% agarose gel electrophoresis, respectively. First-Strand cDNA Synthesis Kit (Eurx, Poland) was used for the synthesis of cDNA. The expression of *TNF-α*, *IL-1β*, *HO-1*, and *NQO-1* genes were evaluated using MIC Real-Time PCR system (Bio Molecular Systems, Australia) and SYBR Green real time Mastermix (Yekta Tajhiz Azma, Iran) at the mRNA level. *GAPDH* was also used as reference gene. Sequence and properties of primers were listed in Table 1. Relative expression levels of the genes were calculated by $2^{-\Delta\Delta Ct}$ [17].

2.6. Western blotting analysis

Kidney tissues were homogenized by Dounce Homogenizer in chilled RIPA (Santa Cruz, USA) buffer containing Protease Inhibitor Cocktail (Sigma, USA). The homogenized samples were vortexed. Then, the samples were shaked using Shaker-Incubator (500 rpm, 4 °C, 2 hr) and vortexed once every 30 minutes in this period. The samples were centrifuged (12000 g, 4°C, 15 min). The supernatants were removed, aliquoted and stored at -70 °C. Lowry method was done to measure protein content. Extracted total protein samples were mixed with sample buffer 3X (2:1) and were put in water bath (95 °C, 5 min). Prepared samples (20 µg protein of each sample) were loaded on discontinues Sodium Dodecyl Sulfate Polyacrylamide Gel (SDS-PAG) and electrophoresed to separate protein bands. The protein bands on the gel were transferred to the PVDF membrane (Santa Cruz, USA). The membrane was blocked by Skim Milk 5% in TBST. Following, primary antibodies were purchased from Santa Cruz: phosphorylated (p)-NF-kB p65 (sc-136548), NF-kB p65 (sc-8008), AMPK (sc-74461) and Nrf2 (sc-28379). Anti-p-AMPK (Thr 172) (#2531) primary antibody was purchased from Cell Signaling Technology (Beverly, USA). Then, the membrane was incubated with horse radish peroxidase (HRP)-conjugated secondary antibody. Finally, target protein bands were developed on X-ray films using with ECL kit (Santa Cruz, USA), and quantified by densitometry using Image J analysis software (version 1.41). β-Actin (Santa Cruz, sc-47778) was used as an internal control for normalization of data.

2.7. Statistical analysis

SPSS package (version 16.0, SPSS) was used for data analysis. Shapiro-Wilk statistical test was done to evaluate normal distribution of data. Then, data were analyzed using One-Way ANOVA or Kruskal-Wallis statistical tests based on the result of Shapiro-Wilk test. Data were reported as mean±SD of three independent replicates. $p<0.05$ was considered as significance level.

3. Results

3.1. Falcarindiol ameliorates kidney function and injury

Cisplatin treatment increased the levels of BUN and serum creatinine in comparison with sham group (62.4±18.3 vs. 25.4±6.2 and 3.7±1.2 vs. 0.85±0.22 mg/dl, p<0.01, respectively). 50 and 100 mg/kg Falcarindiol significantly attenuated the BUN increases in a dose-dependent manner when compared with non-treated cisplatin mice (41.1±12.6 and 33.9±9.5 vs. 62.4±18.3 mg/dl, p<0.05 and p<0.01, respectively). Additionally, the effects of 50 mg/kg Falcarindiol on the serum creatinine concentration was not statistically significant; however, serum creatinine concentration was significantly ameliorated by 100 mg/kg Falcarindiol against the non-treated cisplatin group (1.3±0.3 vs. 3.7±1.2 mg/dl, p<0.01). The administration of alone 100 mg/kg made no considerable changes in the BUN and serum creatinine concentrations in comparison with the sham group (Table 2).

The kidney sections stained with Hematoxylin and eosin (H&E) showed substantial tubular damage accompanied by the infiltration of leukocytes in cisplatin-treated mice. In contrast, sham and Falcarindiol groups demonstrated normal histopathologic features without obvious damage in renal structure. Furthermore, Falcarindiol treatment significantly ameliorated cisplatin-induced damages in a dose-dependent manner (Fig.1, Table 3).

3.2. Falcarindiol mitigates cisplatin-induced nephrotoxicity via the inhibition of NF-kB signaling pathway

For the evaluation of Falcarindiol effects on the renal inflammation, we measured the mRNA expression of TNF-α and IL-1β genes together with protein expression of p-NF-kB p65 using RT-PCR and western blotting, respectively (Fig.2A, 2B and Fig.3A). Cisplatin elevated the mRNA expression of TNF-α and IL-1β (4.23- and 4.84-fold, respectively) accompanied by an increase in the level of p-NF-kB p65 protein (3.8 fold). In contrast, Falcarindiol treatment dose-dependently attenuated cisplatin-induced inflammatory cytokines TNF-α and IL-1β; however, 50 mg/kg Falcarindiol did not make statistically significant alteration on the mRNA expression of IL-1β. In line with RT-PCR results, western blotting showed ameliorating effects for Falcarindiol treatment on the protein level of p-NF-kB p65. These results indicate that Falcarindiol can inhibit the production of pro-inflammatory cytokines via the suppression of NF-kB pathway.

3.3. Falcarindiol mitigates cisplatin-induced nephrotoxicity through the promotion of AMPK and Nrf2 signaling pathways

As shown in Figure 3B, the protein expression of p-AMPK was increased following Falcarindiol administration alone when compared with sham group. Additionally, combination therapy of cisplatin with 100 mg/kg Falcarindiol increased p-AMPK protein level. Moreover, we assessed the Nrf2 signaling pathway through measurement of the mRNA expression of HO-1 and NQO-1 along with protein level of nuclear Nrf2 by using RT-PCR and western blotting, respectively. Falcarindiol alone or in combination with cisplatin elevated protein expression of nuclear Nrf2, consistent with increased expression of Nrf2 signaling target genes including HO-1 and NQO-1 when compared with sham and cisplatin groups. These findings represent the ability of Falcarindiol compound to up-regulate Nrf2 as an antioxidant signaling (Fig.2C, 2D and Fig.3C).

4. Discussion

The present study demonstrated for the first time that Falcarindiol (FAD), a natural polyacetylene compound commonly distributed in the Apiaceae species such as carrot, ameliorated kidney function in cisplatin-induced nephrotoxic mice. FAD mitigated the elevated levels of BUN and creatinine in cisplatin-treated mice, and also improved kidney injury especially tubular damage and leukocytes infiltration. Additionally, FAD exhibited anti-inflammatory and antioxidant properties through the modulation of NF-kB and Nrf2 signaling pathways, respectively.

Cisplatin is one of the most effective drugs against various solid tumors; however, it is well-known that cisplatin causes nephrotoxicity as a major side effect in dose-dependent manner. Nephrotoxicity is observed in approximately one-third of all patients that receive chemotherapeutic cisplatin. Thus, it is important to develop novel agents for alleviating this complication. Several mechanisms are attributed to this condition including DNA damage, inflammation, oxidative stress, and etc. [18, 19]. We previously evaluated the efficacy of two chemical agents to reduce cisplatin-induced nephrotoxicity via the promotion of antioxidant system and the inhibition of renal inflammation [2, 20]. Although there are numerous studies about this issue, more researches are still needed to explore the best chemical agent with minimal toxicity.

The AMP-activated protein kinase (AMPK) is a master regulator of cellular energy homeostasis. The active form of AMPK (phospho-AMPKαThr172) maintains normal tissues from cisplatin-mediated toxicities. AMPK controls many different cellular processes such as inflammation and oxidative stress through modulating down-stream NF-κB and Nrf2 pathways, respectively [21]. These two main pathways play pivotal roles in the regulation of cellular inflammation and the balance between oxidant-antioxidant status. Some studies revealed that AMPK activation induces Nrf2 translocation from cytoplasm into nucleus and thereby augments its respective target genes, which are associated with antioxidant response. Meanwhile, it is indicated that activated AMPK

indirectly downregulates NF-κB signaling. AMPK stimulates sirtuin1, a deacetylase enzyme that removes an acetyl group from lysine residue of NF-kB-p65 subunit, in which subsequently triggers NF-kB-p65 ubiquitination and proteasomal degradation [21, 22].

Some investigations reported that FAD has anti-bacterial activity, and successfully inhibited the infections induced by *Micrococcus luteus*, *Bacillus cereus* and *Pseudomonas aeruginosa*. These findings indicate that FAD can be used as a drug candidate for treating infections [13, 23]. Moreover, FAD exhibited the inhibitory effects on lipopolysaccharide (LPS)-triggered inflammatory molecules production without significant cytotoxicity in colon epithelial cells so that it reduced inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, IL-8 accompanied by an increase in the level of anti-inflammatory cytokine IL-10 [24]. Similar findings were obtained in murine macrophages, and FAD compound attenuated LPS-induced pro-inflammatory cytokines including TNF-α, IL-6, IL-1β and iNOS through protect MAPK and JAK-STAT pathways. However, FAD caused no significant alteration on LPS-stimulated activation of NF-kB and p38 pathways [25]. Additionally, FAD diminished NF-κβ and its respective inflammatory cytokines IL-6, TNFα, and COX-2 in colorectal neoplastic lesions obtained from rats [15]. In line with previous studies, we demonstrated that FAD administration declined mRNA expression of IL-1β and TNF-α together with p-NF-kB p65 protein level in cisplatin nephrotoxic mice.

Some studies indicated the ability of FAD compound to activate Nrf2/ARE signaling. In cell culture experiment, FAD provoked the activation of antioxidant enzymes, such as catalase and NAD(P)H:quinone oxidoreductase 1 (NQO-1) as a drug-metabolizing enzyme via the Nrf2/ARE pathway [26]. FAD can covalently alter the Cys151 residue in Keap1 protein through S-alkylation reaction, and consequently inactive it. Keap-1 traps Nrf2 protein in the cytoplasm, and also stimulates its ubiquitination and proteasomal degradation. Therefore, Keap1 inactivation by FAD stimulates Nrf2 translocation into nucleus, and binding to ARE which results in up-regulation of antioxidant enzymes [14]. Furthermore, it is shown that FAD treatment prevented carbon tetrachloride (CCl4)-induced hepatotoxicity through increasing the glutathione S-transferase (GST) and NQO-1 activities. FAD declined serum concentration of ALT/AST along with lipid peroxidation products in liver tissues of CCl4-treated hepatotoxic mice [16]. In agreement with these findings, our experiments revealed that FAD pre-treatment up-regulated NQO-1 and HO-1 mRNA expression accompanied by an increase in protein levels of nuclear Nrf2 and p-AMPK (upstream regulator of Nrf2) in kidney tissues of nephrotoxic mice. These findings suggest that FAD is a stimulator of antioxidant pathways.

Some studies demonstrated anti-cancer effects for FAD compound. FAD significantly inhibited cell growth and proliferation in different cancer types such as colorectal cancer, breast cancer, hepatocellular carcinoma (HCC) and glioblastoma cells [12, 27-30]. FAD led to cell death through the induction of apoptosis, autophagy and endoplasmic reticulum stress. It is important to note that FAD did not interfere with cisplatin efficiency, and interestingly had synergistic effects in

combination with cisplatin to suppress HCC cells [29]. These results emphasize on the anti-cancer potential of FAD in addition to its anti-inflammatory and antioxidant capabilities; Nevertheless, the effects of FAD treatment on normal stem cells have not been fully evaluated yet; for example, one study exhibited deleterious effects for FAD on the neural stem cells in which impairs the balance between self-renewal and differentiation status [30].

Taken together, our results indicate that FAD ameliorated kidney injury in cisplatin nephrotoxic mice thorough down-regulation of NF-kB signaling pathway and its respective inflammatory cytokines such as TNF-α and IL-1β, as well as up-regulation of AMPK-Nrf2-ARE axis which stimulates antioxidant enzymes such as HO-1 and NQO-1. According to our results, FAD can be used clinically for the alleviation of nephrotoxicity in patients who receive cisplatin; however, further researches will be needed to clarify the effects of FAD on normal tissues homeostasis.

5. Conclusion

The anti-cancer natural compound Falcarindiol represents therapeutic potential due to its anti-inflammatory and antioxidant properties via the modulation of NF-kB and Nrf2 pathways in cisplatin induced nephrotoxic mice.

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Data availability

Data will be made available on request.

Conflict of interest The authors declare no competing interests.

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Figures and Tables legends

Fig.1 The kidney damage was semi-quantitatively scored between 0 to 5 of 10 fields/slide at 400X magnification (0= indicating normal; 1= 1-10%; 2= 11- 25%; 3= 26-50%; 4= 51-75%; 5= 76-100%). **Group 1,(Sham):** healthy control mice receiving vehicle of Cisplatin and Falcarindiol; **Group 2, FAL(100mg/kg)**: mice received Falcarindiol 100 mg/kg; **Group 3, Cis(15mg/kg)**: Cisplatin-induced nephrotoxic control group; **Group 4, Cis(15mg/kg) + FAL(50mg/kg**: Cisplatin-induced nephrotoxic mice treated with 50 mg/kg Falcarindiol; **Group 5, Cis(15mg/kg) + FAL(100mg/kg)**: Cisplatin-induced nephrotoxic mice treated with 100 mg/kg Falcarindiol; Data are presented as mean±SD of three independent replicates (N=6). *p<0.05 *vs.* Sham; **p<0.01 vs. Sham; #p<0.05 *vs.* Cis; and ##p<0.01 vs. Cis.

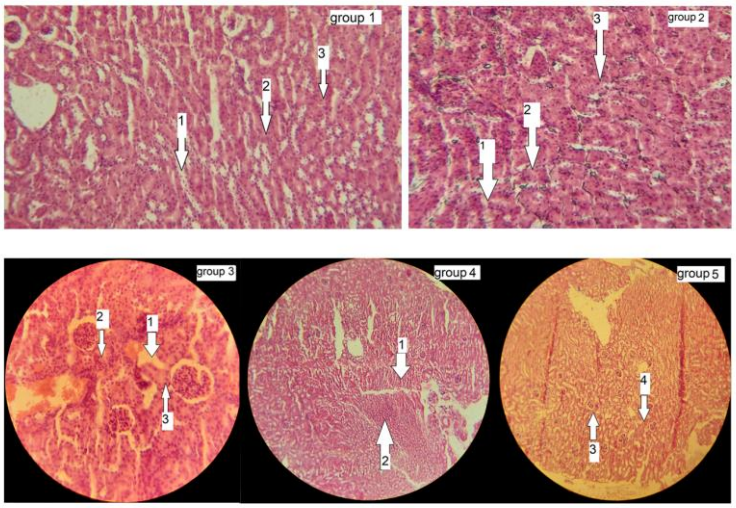

Fig.2 The effects of Falcarindiol on the mRNA expression of TNF-α (A), IL-1β (B), HO-1 (C), and NQO-1 (D) as evaluated by Real-time PCR. Sham: healthy control mice receiving vehicle of Cisplatin and Falcarindiol; Cis: Cisplatin-induced nephrotoxic control group; Cis + 50 FAL: Cisplatin-induced nephrotoxic mice treated with 50 mg/kg Falcarindiol; Cis + 100 FAL: Cisplatin-induced nephrotoxic mice treated with 100 mg/kg Falcarindiol; 100 FAL: healthy mice receiving only 100 mg/kg of Falcarindiol. ****p<0.0001 vs. Sham; #p<0.05, ##p<0.01, ###p<0.001, and ####p<0.0001 vs. Cis.

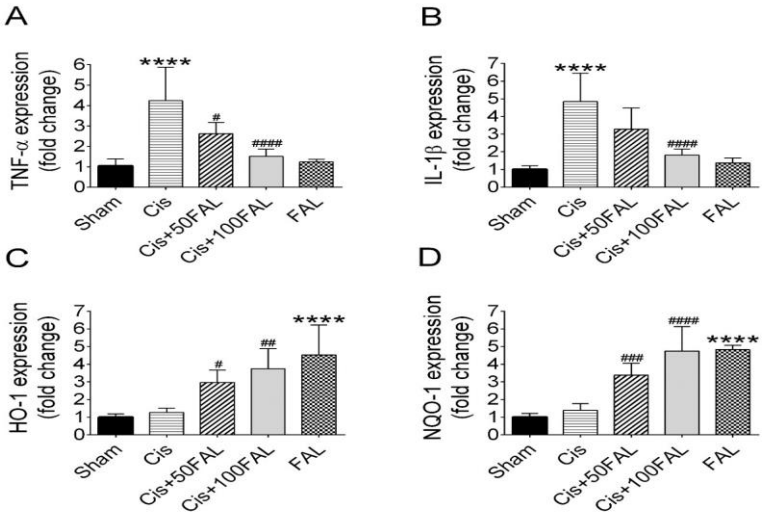

Fig.3 The effects of Falcarindiol on the protein level of p-NF-kB P65 (A), p-AMPK (B) and nuclear Nrf2 (C) in which determined by Western blotting. Sham: healthy control mice receiving vehicle of Cisplatin and Falcarindiol; Cis: Cisplatin-induced nephrotoxic control group that received 15 mg/kg of Cisplatin; Cis + 50 FAL: Cisplatin-induced nephrotoxic mice treated with 50 mg/kg Falcarindiol; Cis + 100 FAL: Cisplatin-induced nephrotoxic mice treated with 100 mg/kg Falcarindiol; 100 FAL: healthy mice receiving only 100 mg/kg of Falcarindiol. *p<0.05, **p<0.01, and ****p<0.0001 vs. Sham; #p<0.05, ##p<0.01, and ####p<0.0001 vs. Cis.

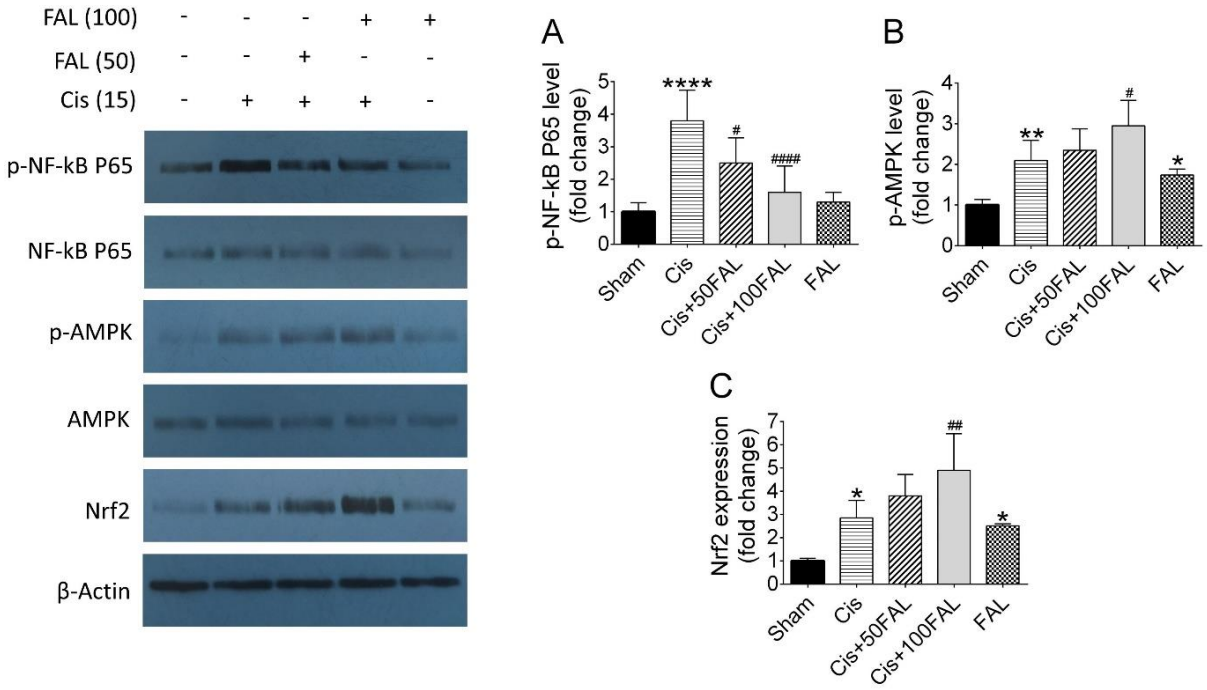

Table 1. List of primers and their respective characteristics.

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)	NM accession number	Product length
TNF-α	ACCACGCTCTTCTG TCTACTG	CTTGGTGGTTTGCT ACGAC	013693.3	169
IL-1β	TCCCATTAGACAAC TGCACTAC	GCTCATGGAGAATAT CACTTGTTG	008361.4	133
HO-1	CAACATTGAGCTGT TTGAGGAG	GTGTCTGGGATGAG CTAGTG	010442.2	173
NQO-1	ACCTGGTGATATTT CAGTTCCC	AGTGGTGATAGAAAG CAAGGTC	008706.5	161
GAPDH	TGAACGGATTTGGC CGTATTG	CTTGACTGTGCCGT TGAATTTG	001289726.1	161

Table 2. Kidney function tests.

	Sham (N=6)	Cis (N=6)	Cis + 50 FAL (N=6)	Cis + 100 FAL (N=6)	100 FAL (N=6)
BUN (mg/dl)	25.4±6.2	62.4±18.3**	41.1±12.6#	33.9±9.5##	26.5±7.8
Scr (mg/dl)	0.85±0.22	3.7±1.2**	2.5±0.8	1.3±0.3##	0.9±0.28

Table 3. Histopathologic evaluation of kidney injury in mice.

	Sham	**FAL(100mg/ml)**	**Cis(15 mg/kg)**	**Cis(15 mg/kg) + FAL (50 mg/kg)**	**Cis(15 mg/kg) + FAL (100mg/ml)**
Glomerular injury	0.25±0.09	0.36±0.12	2.53±0.81*	2.23±0.73	1.65±0.54#
Tubular damage	0.3±0.13	0.62±0.21	4.21±0.62**	3.44±0.76#	2.31±0.78##
Leukocytes infiltration	0.42±0.16	0.67±0.34	3.87±0.78**	3.24±0.53#	2.53±0.83##
Interstitial damage	0.17±0.06	0.18±0.07	2.07±0.43*	1.73±0.63	1.32±0.48#
Vascular damage	0.09±0.03	0.14±0.08	1.86±0.31*	1.58±0.47	1.49±0.69#

Table.3 Sham: healthy control mice receiving vehicle of Cisplatin and Falcarindiol; Cis: Cisplatin-induced nephrotoxic control group; Cis + 50 FAL: Cisplatin-induced nephrotoxic mice treated with 50 mg/kg Falcarindiol; Cis + 100 FAL: Cisplatin-induced nephrotoxic mice treated with 100 mg/kg Falcarindiol; 100 FAL: healthy mice receiving only 100 mg/kg of Falcarindiol. BUN: blood urea nitrogen; Scr: serum creatinine. Values are presented as mean±SD of three independent replicates (6mice/group). **p<0.01 *vs.* Sham; #p<0.05, ##p<0.01 *vs.* Cis