Original Article

Nicorandil Mitigates Renal Oxidative Damage, Apoptotic, and Fibrotic Changes Induced by Cyclosporine-A in Rats

Heba M. Hafez[1], Mervat Z. Mohamed[1*], Hend Abdel-Ghany[2], Nagwa M. Zenhom[2], Manal A.Khalaf[3], and Maram Elhussieny[3]

[1]Department of Pharmacology, Faculty of Medicine, Minia University, 61511 Minia, Egypt.
[2]Department of Biochemistry, Faculty of Medicine, Minia University, 61511 Minia, Egypt.
[3]Department of Pathology, Faculty of Medicine, Minia University, 61511 Minia, Egypt.

A B S T R A C T

Cyclosporine A (CsA) is one of the immunosuppressive drugs broadly used in transplantation rejection. However, nephrotoxicity arising from its long-term use constrained its efficacy. This study investigated the advantageous role of nicorandil against CsA nephrotoxicity in rats. Rats were assigned into 5 groups (n= 6 each): control group, nicorandil group, CsA group, CsA+nicorandil group, CsA+nicorandil+glibenclamide group. CsA induced Nephrotoxicity orally for 21 days. The results exhibited that CsA nephrotoxicity manifested by increasing serum urea, creatinine, and uric acid in the orchestra with histopathological changes. There was a significant rise in oxidative damage parameters;malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) without relevant effect on nitric oxide (NO) level. CsA increased the expression of transforming growth factor beta (TGF-β) and collagen 1A1 by reverse transcriptase (RT-PCR) method with reduction of immunohistochemical expression of the Bcl2 level and increasing caspase-3 level. Administration of nicorandil significantly amelioratedoxidative changes with elevation in NO level. Nicorandil prevented the elevated serum markers associated with significant improvement in TGF-β, collagen IA1 and endothelial nitric oxide synthase (eNOS) expression. Besides,immunohistochemical expression of Bcl2 significantly augmented with a reduction in caspase-3 in comparison with CsA. Glibenclamidedid not significantly have a role when combined with CsA and nicorandil. The renal protective effect of nicorandil was thought to be in part through anti-oxidant, anti-fibrotic, and anti-apoptotic mechanisms. These results may offer additional nephroprotective mechanisms for nicorandil independent on its vasodilator effect.

Key Words: Cyclosporine; nicorandil; nephrotoxicity; glibenclamide.

Corresponding Author: *Mervat Zaki Mohamed*   **Email:** *mervat_jana@yahoo.com*

1. INTRODUCTION

Cyclosporine-A (CsA) is a calcineurin inhibitor applied widely as the immunosuppressive agent in organ transplantation and autoimmune illnesses (Halloran, 1996; Yong-Gang et al., 2014). However, its therapeutic benefits are often limited by serious organ toxicity; the most important of which is nephrotoxicity (Takasu et al., 2015). It was suggested that the possible mechanisms underlying short- and long-term nephrotoxicity induced by CsAis atrophy, fibrosis, inflammation, and sclerosis. Several factors contributed; one of the main causative factors is the increased generation of free radical species and lipid peroxidation products (Sereno et al., 2014, Ateyya, 2015). The pathogenesis of CsA toxicity on renal tissues comprises afferent arteriolar vasoconstriction with pro-fibrotic effect in its chronic stage and inhibition of intra-renal nitric oxide (NO)(Mazali et al.,

2012). Renal damage progresses through activation of the immune mediators such astransforming growth factor(TGF-β) which is known to be an important cytokine in the pathogenesis of glomerulosclerosis and interstitial fibrosis in CsA toxicity (Kim et al., 2016). Deregulation of apoptosis has been involved additionally in the pathogenesis of CsA-induced nephrotoxicity by promoting caspase-3 activation (Lai et al., 2015). Nicorandil an ATP-sensitive potassium channel opener with NO donation property has been shown to have a significant protective effect against ischemic heart diseases; it exhibits vasodilation in the vascular system (Zhang et al., 2015). Former reports demonstrated renal defense in mesangioproliferative glomerulonephritis, and ischemia reperfusion injury but its role in CsA nephrotoxicity was not investigated (Sudo et al., 2009). Beneficial effects of nicorandil have been reported in various organs that were linked to its anti-oxidant and

anti-proliferative changes (Tanabe et al., 2012).The aim of the current study was to evaluate the possible protective role of nicorandil on chronic nephrotoxicity induced by CsA exploring putative antioxidant, anti-fibrotic, and anti-apoptotic mechanisms.

2. MATERIALS AND METHODS

2.1. Animals

The present research was conducted on adult male albino rats weighing 150–200 g. Rats were gotten from the National Research Center, El-Giza, Egypt. Rats were permitted an ordinary diet of commercial rat food and water and were adjusted to laboratory conditions for a period of 2 weeks. The experiments were done in accordance with the Guide for the Care and Use of Laboratory Animals of the National research council. The protocol was accepted by the Faculty of Medicine, Minia University Ethics Committee for the Care and Use of Laboratory Animals.

2.2. Chemicals and antibodies

Cyclosporine A (Sandimmun® soft gelatin capsules; 50 mg/ml) was obtained from Novartis (Basel, Switzerland) and dissolved in olive oil. Nicorandil was purchased from (Merck Ltd., Cairo, Egypt) dissolved in distilled water. Anti-Bcl2 and caspase-3 polyclonal antibodies were purchased from Thermo Fisher Scientific Inc. /Lab Vision (Fermont, CA, USA).

2.3. Experimental design

The rats were assigned randomly into 5 groups ($n = 6$ at each group) and treated every day for 21 days. Group 1 served as control group and received (1ml/rat/day, p.o.) olive oil (vehicle). Group 2: Rats were given CsA (25 mg/kg, p.o.) diluted in olive oil (Chandramohan and Parameswari, 2013). Group 3: Rats were treated with nicorandil (15 mg/kg, p.o.) dissolved in distilled water (Elshazly, 2015). Group 4: Rats received the formerlystated doses of CsA combined with nicorandil for a period of 21 days. Group 5 were given the same regimen as Group 4 together with glibenclamide (5 mg/kg/day, p.o.) (Sanada et al., 2002)2 h before nicorandil administration.

At the termination of experimental period (21 days), rats were fasted for 12 h and urethane was used for anesthesia. Blood was collected after decapitation and centrifuged at 5000 rpm for 15 min for sera. Sera were saved at -20 °C until assessment of urea, creatinine, and uric acid. Both kidneys were removed, washed with physiological saline, and dried on filter paper. Two half parts were taken for immunohistochemical and PCR screening and the remaining were homogenized in ice-cold saline and kept at -80 °C for measurement of biochemical parameters.

2.4. Biochemical analysis

2.4.1. Measurement of renal functions

Serum urea, creatinine, and uric acid were estimated by enzymatic colorimetric kits according to previous techniques (Fawcett and Scott, 1960; Schirmeister et al., 1964).

2.4.2. Measurements of oxidative stress parameters

2.4.2.1. Lipid peroxidation product

MDA, a lipid peroxidation indicator, was analyzed by a spectrophotometric method that built on a reaction with thiobarbituric acid (Buege and Aust, 1978). Colored complexes were obtained when extracted with n butanol/pyridine. Sample absorbance was measured at 532 nm and calculated using the absorbance of the standard using 1, 1, 3, 3-tetramethoxy propane.

2.4.2.2. Measurement of nitric oxide in renal tissue

Nitric oxide production was measured by levels of total nitrite and nitrate (NO_2^- and NO_3^-) as indicators. Total nitrite and nitrate were estimated after the reduction of nitrate to nitrite by copperized cadmium particles in glycine buffer at pH 9.7. A concentration of NO_2^- was based on the Griess reaction, in which a color substance with absorbance at 540 nm is formed by reaction of nitrite with a mixture of naphthylethylenediamine and sulfanilamide, method described by Ridnouret al. (Ridnour et al., 2000). Sodium nitrite and nitrate solutions were used for standard measurements.

2.4.2.3. Renal SOD activity

It was measured in accordance with the method described by (Marklund and Marklund, 1974) with a minor modification. This method is based on inhibition of pyrogallol autoxidation by SOD which activity was estimated at 420 nm.

2.4.2.4. Measurement of GSH in renal tissue

According to Moron et al. (1979) method,GSH was estimated. The reaction mixture including 0.25 mL of supernatant, 1 mL of 0.2 M Tris-HCl (containing 1mM EDTA, pH 8.9), and 0.05 mL of 0.01 M 5,5-dithiobis- (2-nitrobenzoic acid) in absolute methanol was preserved at room temperature for 5 min. Yellow color was measured spectrophotometrically at 412 nm. Glutathione concentration in tissue

$$= \frac{\text{A sample} \times 66.66}{\text{g. tissue used}} \text{ mg/g tissue}$$

2.5. Reverse transcriptase (RT-PCR) analysis for detection of TGF-β, collagen 1A1 and eNOS mRNA expression

Total RNA was purified from homogenized kidney tissues using RiboZol RNA Extraction reagent

(Amresco, Solon, USA) following the manufacturer's instructions. Nucleic acid concentrations were determined by spectrophotometer (Genova Plus, Bibby Scientific, UK). Reaction tube of RT-PCR was containing 5 μl of RT-PCR buffer 5×, 10 pM of specific primers for TGF-β, Coll1A1, eNOS and GAPDH, 5 μg of total RNA template, 1 μl dNTPs mix (100 mM), 1 μl enzyme mix (Hot Star Taq DNA polymerase, Omniscript and Sensiscript reverse transcriptases). PCR amplification was performed in a DNA thermal cycler (Progene; Techne Ltd., Duxford, United Kingdom). Rat TGF-β PCR amplification was performed as follow: 5 min, at 95°C (initial denaturation); 94°C for 20 s; 51°C for 30 s; 72°C for 40 s (30 cycles); and 72°C for 5 min (final extension). The $MgCl_2$ concentration used for cDNA amplification was 2.0 mM for rat TGF-β. The RT-PCR condition for rat Coll1A1, rat eNOS and GAPDH was the same as rat TGF-β except that the annealing temperature was 50°C, 55 °C and 54°C respectively. Primers used and amplicon size are as follows, rat TGF-β, Sense: 5' TTGCCCTCTACAACCAACACAA 3' and Antisense, 5' GCTTGCGACCCACGTAGTA 3'; 103bp. rat Coll1A1 Sense: 5' TTCCCTGGACCTAAGGGTACT 3' and Antisense: 5' TTGAGCTCCAGCTTCGCC 3'; 114bp. rat eNOS sense: 5'-CGA GATATC TTC AGT CCC AAG C-3' and antisense 5'-GTG GAT TTG CTG CTC TCT AGG-3' 135bp. PCR was performed with rat GAPDH as an internal standard (Thermo scientific) USA. Sense5'-CAGTGCCAGCCTCGTCTCAT-3', antisense 5'-GGGGCCATCCACA GTCTTC-3, 595bp'.The intensity of the PCR product bands were quantified using gel documentation system software (Biometra GmbH, Germany).

2.6. Detection of PCR Product

Polymerase chain reaction products (5 μl) were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Location of a predicted product was confirmed by using 100 bp ladder (Gene Ruler, Thermo scientific, USA)) as a standard size marker.

2.7. Histopathological changes in renal tissues

The kidney specimens were fixed in 10% formalin for at least 24 h. Then, kidney tissues were dehydrated with a sequence of ethanol solutions, embedded in paraffin, cut into 5 μm thickness, and stained with hematoxylin and eosin dye. These sections were then examined by a pathologist for randomly selected 10 fields from each rat.The histopathological scoring analysis was performed, the assessment was expressed as the sum of the individual score grades from 0 (no findings), 1 (mild), 2 (moderate), to 3 (severe) for each of the following 4 parameters from kidney sections: tubular cell swelling, cellular vacuolization, pyknotic nuclei and medullary congestion (Singh and Chopra, 2004).

2.8. Immunohistochemical localization of Bcl2and caspase-3 proteins in renal tissue

Five μm sections placed on positively charged slides (CITOGLAS) were prepared. Sections were de-paraffinized with xylene, hydrated through 99.9%, 95%, and 70% ethanol.For antigen retrieval, sections were treated for 20 minutes in citrate buffer (pH 6.0) by microwave, and then allow cooling. The slides then treated with 3% hydrogen peroxide for 30 minutes to inactivate endogenous peroxides, washed in phosphate-buffered saline (PBS) solution. Primary antibodies were incubated overnight in a humidity chamber using caspase-3 and Bcl2 primary antibodies (Polyclonal rabbit antibody, 7ml Ready to use, Lab Vision Laboratories), then washed with PBS before applying the biotinylated secondary antibody (Lab Vision Laboratories) for 30 min. Sections washed in PBS, incubated with the streptavidin-biotin complex reagent (Lab Vision Laboratories) for 30 min. A brown color was developed with 3, 3-diaminobenzidinetetra hydrochloride (DAB, Lab Vision Laboratories) for 5 min, then washed in distilled water, counterstained with Mayer's haematoxylin, dehydrated, cleared in xylene, mounted and covered slipped.

2.8.1. Scoring of immunostaining

Screening of sections was done under light microscope magnification X200. Positivity for Bcl2protein family occurs in the cytoplasm. The immunoexpression of Bcl2 was evaluated based on stain intensity, scored from 0 to 3, with 0 considered negative, 1 as weak, 2 as intermediate and 3 as strong. The number of positive cells was evaluated on a scale of 0 to 3, where 0 corresponded to 0 to 10% of cells, 1= 11 to 25%, 2 = 26 to 50% and 3 = more than 51% of cells. A score was obtained for each reaction by multiplying the intensity of the reaction by the percentage of positive cells (Krajewska et al., 1996). Regarding caspase-3, tissue section considered positive when > 10% of the cells displayed cytoplasmic staining (Zhang et al., 2009).

2.9. Statistical analysis

Data were presented as the means ± S.E.M. The results were analyzed using the One-way ANOVA followed by Tukey's multiple comparison tests. Statistical analysis was performed using Graph Pad Prism software (version 5). Differences with P value < 0.05 were considered significant.

3. RESULTS

3.1. Effect of nicorandil and CsA on renal function

CsA administration significantly increased serum urea, creatinine and uric acid as compared with control group. These parameters were significantly improvedin CsA+nicorandil and CsA+ nicorandil+glibenclamide as compared with CsA (Table 1).

3.2. Effect of nicorandil on CsA induced nitrosative stress

Administration of CsA didn't significantly affect NO level in renal tissue as compared with control group. Treatment with nicorandil in CsA intoxicated rats increased NO level significantly compared with control(Table 2).

3.3. Effect of nicorandil on CsA induced oxidative changes

CsA-intoxicated rats showed significant elevation in MDA level and reduction in SOD and GSH activities in renal tissue as compared with control group. CsA-treated group either with nicorandil or with nicorandil+glibenclamide significantly decreased MDA or increased GSH as compared with CsA (Table 2).SOD activity was raised only in CsA+ nicorandil compared with CsA.

3.4. Effect on renal histopathological changes

Kidney of rats intoxicated with CsA showed evident histological changes in the form of epithelial cell swelling and vacuolization with many pyknotic nuclei and medullary congestion. Treatment with nicorandil either alone or in combination with glibenclamide improved renal damage as compared with CsA (Figure 1).

3.5. Immunohistochemical expression of Bcl2 and caspase-3 proteins in renal tissue

The expression level of Bcl2 protein in renal tissue was markedly decreased in CsA-intoxicatedrats while caspase-3 was increased as compared with control group. However, CsA+ nicorandil and CsA+ nicorandil+glibenclamide groups showed significant increasein expression of Bcl2 and decrease caspase-3 expressionas compared with CsA group (Figure 2 and 3).

3.6. RT-PCR localization of TGF-β, Collagen IA1 and eNOS mRNA in renal tissue

CsA caused significant up-regulation of TGF-β (Figure 4) and Collagen IA1 (Figure 5) expression as well as down-regulation of eNOS expression (Figure 6) in renal tissue in comparison with control groups. There was significant reduction in TGF-β and collagen IA1 with significant elevation in expression level of eNOS inCsA+ nicorandil and CsA+ nicorandil+glibenclamide groups as compared with CsA.

Table (1): Effect of cyclosporine A (CsA) and nicorandil on serum renal function

Groups	Urea (mg/dl)	Creatinine (mg/dl)	Uric acid (mg/dl)
Control	38.84±3.39	0.54±0.02	1.50±0.08
Nicorandil	29.60±2.08	0.54±0.02	1.40±0.05
CsA	62.33±3.44[a]	1.00±0.08[a]	3.81±0.09[a]
Nicorandil + CsA	30.20±2.45[b]	0.58±0.01[b]	1.80±0.07[b]
Nicorandil+glibenclamide+CsA	28.17±2.73[b]	0.68±0.03[b]	1.50±0.03[b]

All parameters are expressed as means ± S.E.M. (n=5). [a, b] significantly different (at $P< 0.05$) from normal control group and CsAgroup, respectively. CsA: cyclosporine

Table (2): Effect of Cyclosporine (CsA) and nicorandil on oxidative stress parameters in renal tissue

Groups	MDA (nmol/g tissue)	Nitrate/ nitrite level (nmol/ml)	SOD (U/g tissue)	GSH (mg/g tissue)
Control	101.30±7.34	106.71±4.81	1237.10±56.50	103.20±5.11
Nicorandil	113.51±6.94	275.01±10.82[a]	1129.20±33.49	122.21±4.48
CsA	159.54±11.41[a]	158.80±5.20	445.80±18.25[a]	71.22±2.30[a]
Nicorandil + CsA	120.42±8.47[b]	246.70±4.10[ab]	1193.01±55.28[b]	97.80±3.65[b]
Nicorandil+gliben+CsA	109.22±5.53[b]	252.81±8.21[ab]	754.83±20.35	105.82±4.33[b]

All parameters are expressed as means ± S.E.M. (n=5). [a, b] significantly different (at $P< 0.05$) from normal control group and CsA group, respectively. CsA: cyclosporine; MDA: malondialdehyde; NO: nitric oxide; SOD: superoxide dismutase; GSH: reduced glutathione.
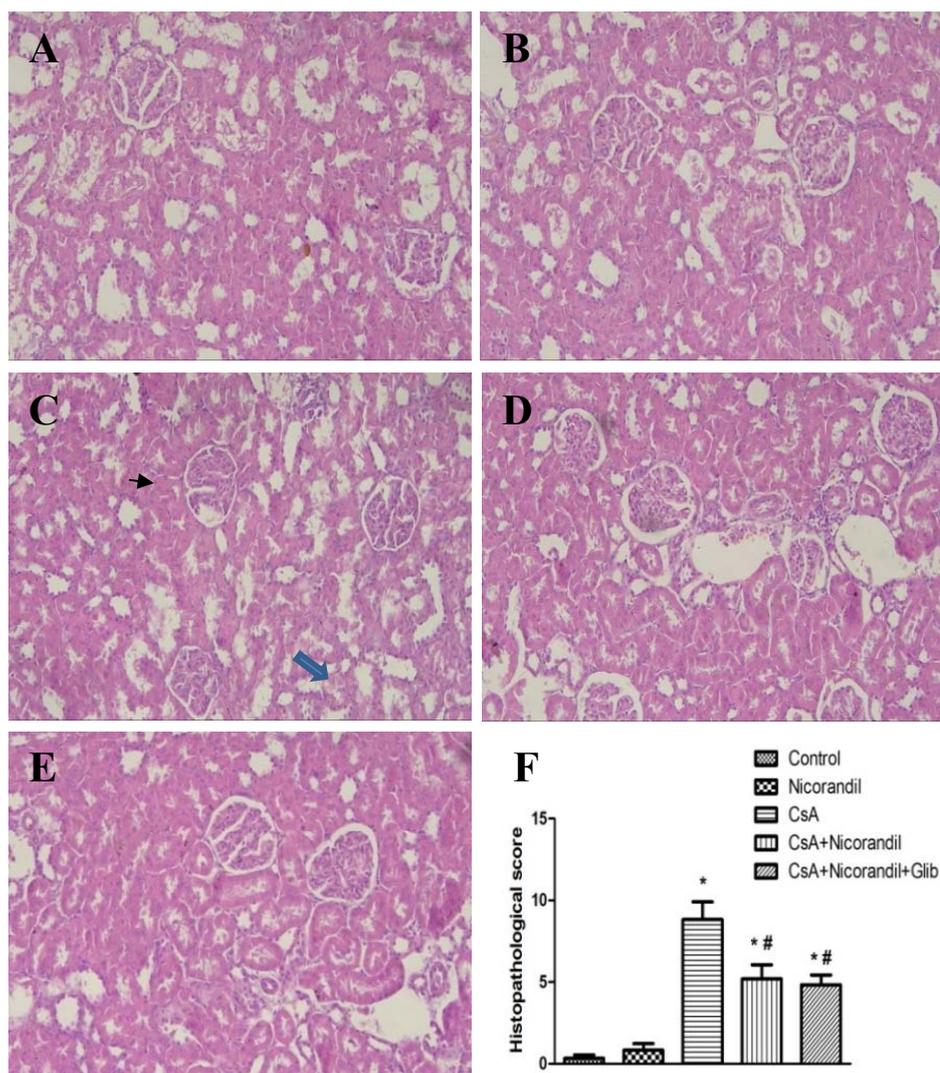
Figure 1: Histopathological findings in renal sections stained with H&E

(A): Control group with normal renal histology. (B): Nicorandil group has normal renal histology. (C): CsA group showed swelling and vacuolization in epithelial cells of renal tubules, the structure of the epithelial cells was unclear. Blue arrow indicates cellular vacuolization, Black arrow cellular swelling. Many pyknotic nuclei and medullary congestion were also observed. (D) and (E): CsA+Nicorandil group and CsA+Nicorandil+glibenclamide group showing histological improvement (Original magnification 100x). (F): Data are expressed as means ± S.E.M. (n = 5). *,# significantly different at ($P < 0.05$) from normal control group and CsA group; respectively. (CsA): cyclosporine; (Glib): glibenclamide.
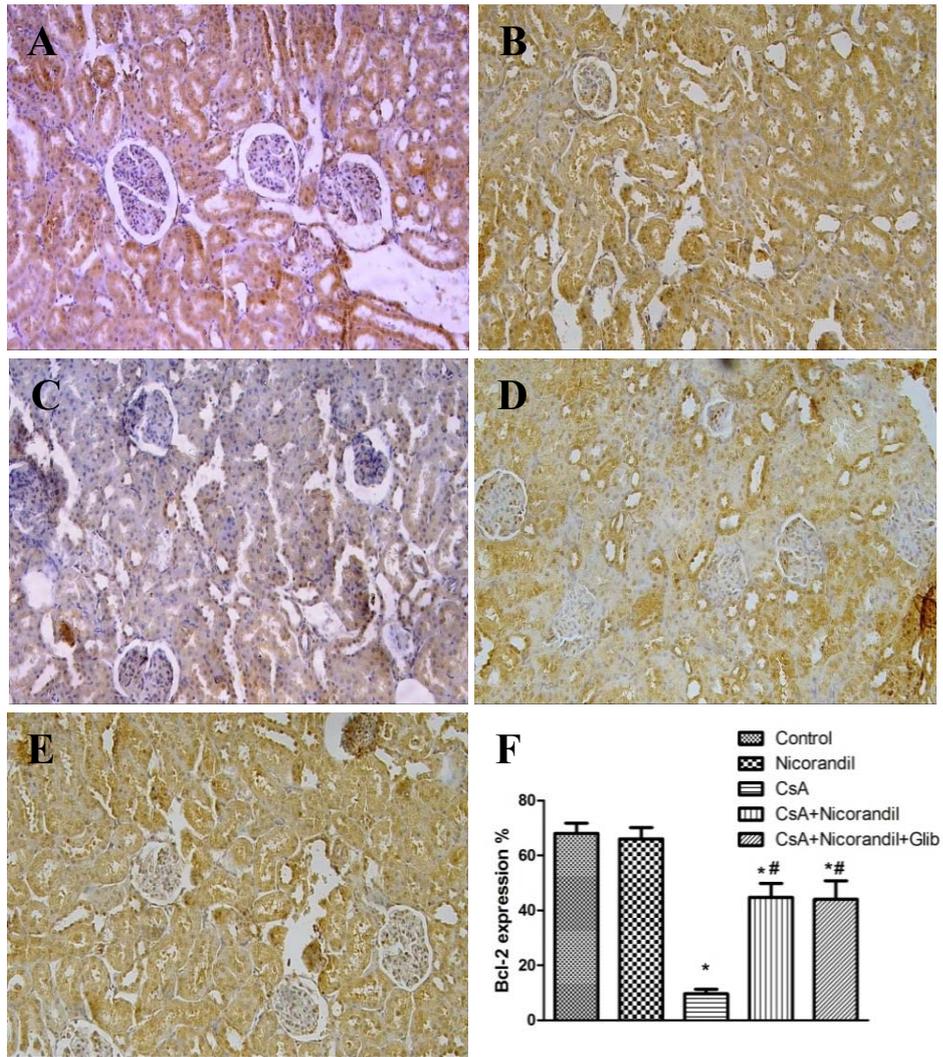
Figure 2: Bcl2 immunostaining results in renal tissue
Reduced expression of Bcl2 was noted in the CsA group (C) when compared with control group (A). Furthermore, a more significant increase in its expression was observed after administration of either nicorandil (D) or with glibenclamide (E) in comparison with CsA. (F): Data are expressed as means ± S.E.M. (n = 5). *,# significantly different at (P < 0.05) from normal control group and CsA group; respectively. (CsA): cyclosporine; (Glib): glibenclamide.
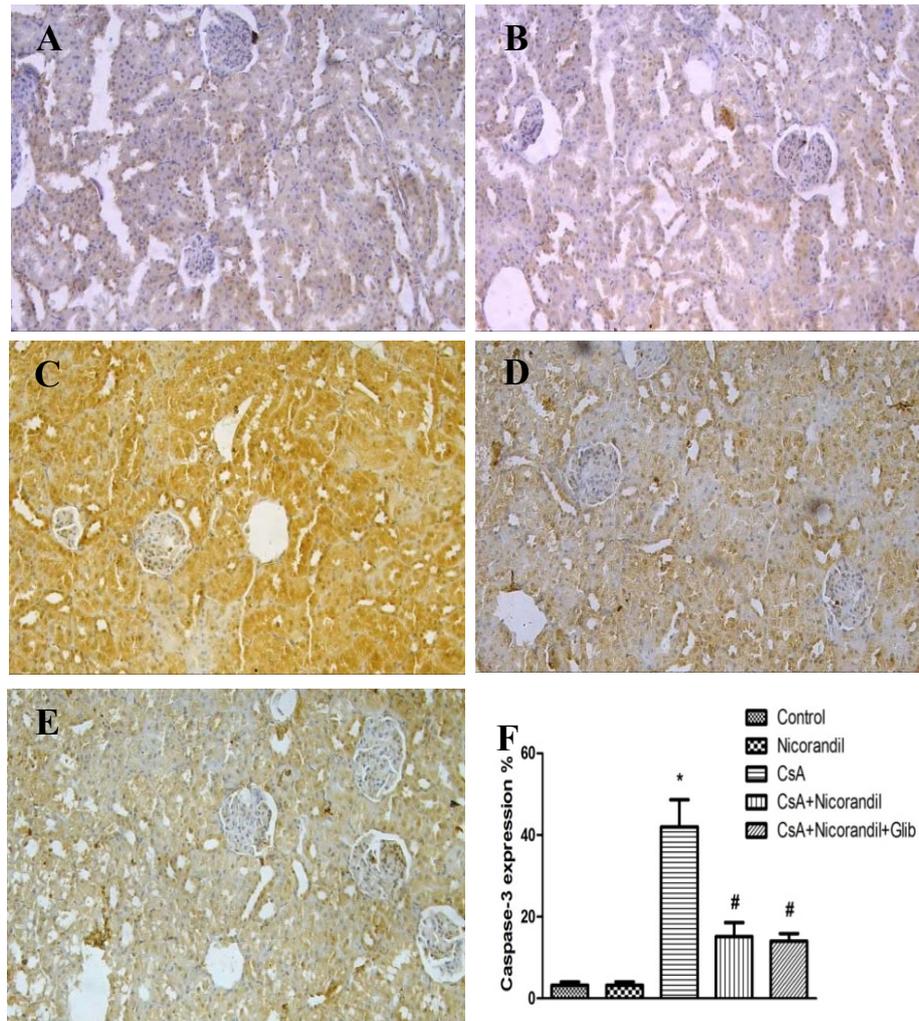
Figure 3: Caspase-3 immunostaining results in renal tissue
(A): Control group show no immunostaining (200x). (B): Nicorandil group with a trivial expression (200x). (C): CsA group showing high immunostaining (200x). (D) and (E): CsA+ Nicorandil group and CsA+Nicorandil+glibenclamide group showing low expression as compared with CsA group (200x). (F): Data are expressed as means ± S.E.M. (n = 5). $^{*, \#}$ significantly different at (P < 0.05) from normal control group and CsA group respectively. (CsA): cyclosporine; (Glib): glibenclamide.
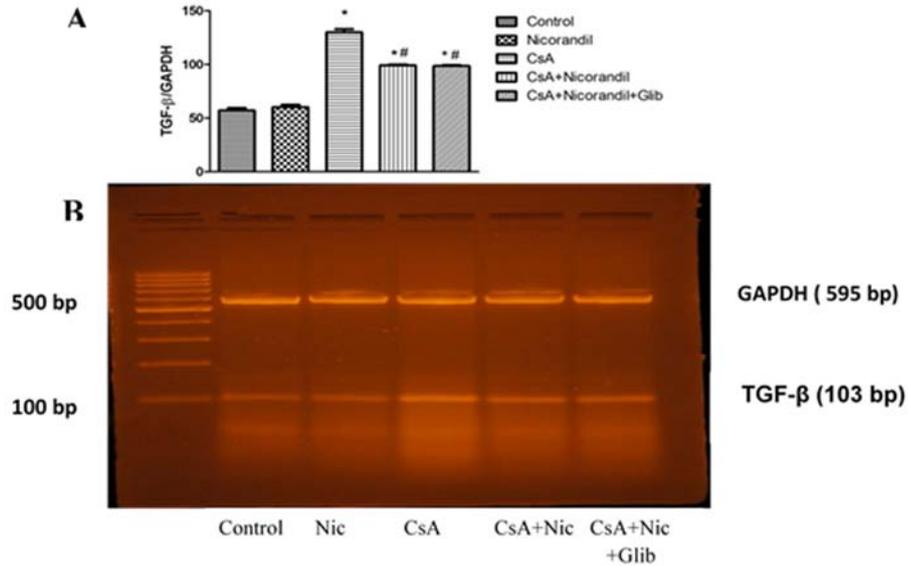
Figure 4: Analysis of TGF-β gene expression. (A, B) represent RT-PCR gel of TGF- β expression. TGF- β mRNA expression was significantly elevated in CsA group compared to control group. There was significant reduction in expression level of TGF-β in CsA groups treated with nicorandil and nicorandil+glibenclamide. Data are expressed as means ± S.E.M. (n = 5). [*,#] significantly different at (P < 0.05) from normal control group and CsA group, respectively. (Nic): nicorandil; (CsA): cyclosporine; (Glib): glibenclamide; (GAPDH): glyceraldehyde-3-phosphate dehydrogenase.
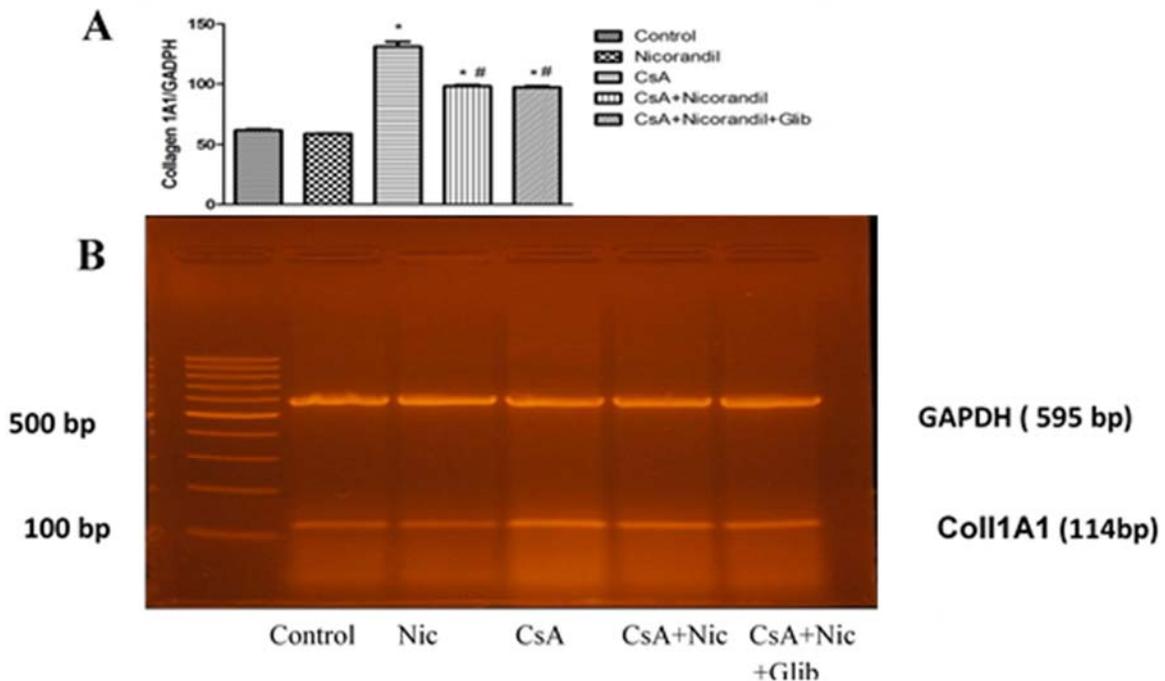

Figure 5: Analysis of collagen 1A1 gene expression. (A, B) represent RT-PCR gel of collagen 1A1 expression. Collagen1A1 mRNA expression was significantly elevated in CsA group compared to control group. There was significant reduction in expression level of collagen 1A1 in CsA groups treated with nicorandil and nicorandil+glibenclamide. Data are expressed as means ± S.E.M. (n = 5). [*,#] significant difference at (P < 0.05) from normal control group and CsA group, respectively. (Nic): nicorandil; (CsA): cyclosporine; (Glib): glibenclamide; (GAPDH): glyceraldehyde-3-phosphate dehydrogenase .
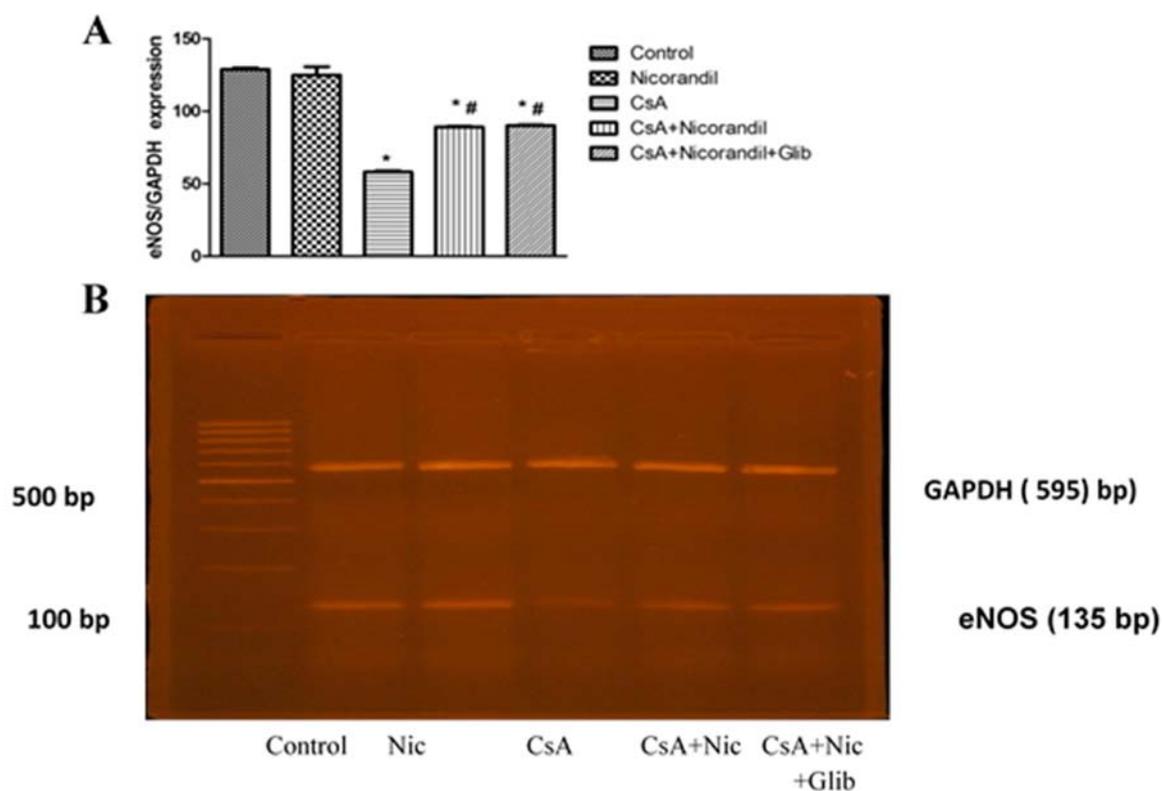
Figure 6: Analysis of eNOS gene expression. (A, B) represent RT-PCR gel of eNOS expression. eNOS mRNA expression was significantly decreased in CsA group compared to control group. There was significant increase in expression level of eNOS in CsA groups treated with nicorandil or nicorandil+glibenclamide. Data are expressed as means ± S.E.M. (n = 5). [*][#] significantly different at (P < 0.05) from normal control group and CsA group, respectively. (Nic): nicorandil; (CsA): cyclosporine; (Glib): glibenclamide; (eNOS): endothelial nitric oxide synthase; (GAPDH): glyceraldehyde-3-phosphate dehydrogenase.

4. DISCUSSION

Nicorandil, a K_{ATP} channel opener, and NO donor could ameliorate CsA toxicity in renal tissue by enhancement of anti-oxidant capacity and damping of inflammatory process as well as decrease pro-fibrotic genes. The results of the present study demonstrated that mechanisms involved in this protection were not likely to couple with activation of K_{ATP} channels. In the present work, CsA induced toxicity on the renal tissue was obvious biochemically and histopathologically. Elevated renal urea and creatinine were in harmony with renal morphological changes in the form of tubular degenerations and vacuolization (Korolczuk et al., 2014; Takasu et al., 2015). Furthermore, CsA-induced hyperuricemia was observed in the current study. Development of hyperuricemia may be secondary to a reduction in uric acid excretion that mediates an ischemic vasoconstriction and acts as a pro-oxidant; playing a deleterious role in renal diseases (Mazali et al., 2012; Tamura et al., 2012).Nicorandil offered a significant improvement in renal function and morphology in CsA-intoxicated rats. This was supported by other clinical and experimental studies, which demonstrated its beneficial role in renal insufficiency disorders (Shiraishi et al., 2014; Fan et al., 2016) and renal ischemia reperfusion injury (Shimizu et al., 2011). Mechanisms underlying CsA nephrotoxicity are still not clarified, however; oxygen free radicals still one of the injurious factors. The role of oxidative damage by CsA on the renal tissue was clear in the present study. CsA induced elevation of lipid peroxidation product, MDA, and reduction of antioxidant defense system GSH and SOD levels. Previous studies related CsA-induced reactive oxygen species by NADPH and xanthine oxidases, with cellular injury and reduction in GSH and SOD (Takasu et al., 2015). Anti-oxidant enzymes GSH and SOD are considered major defense mechanisms against oxidative stress associated with CsA administration (Sereno et al., 2014; Korolczuk et al., 2014).In the present work, nicorandil provides its reno-protective effect in part through amelioration of oxidative stress and increasing anti-oxidant system together with indirect action on pro-oxidant substances such as uric acid level, this was supported by previous studies in different organs (Shiraishi et al., 2014; Elshazly, 2015). Our finding revealed no detectable effect for glibenclamide, a K_{ATP} channels blocker, on resultant

nicorandil protection against CsA nephrotoxicity. That is may exclude the possible role of potassium channel activation.Complementary, the role of NO in both CsA toxicity and protection was further examined. NO is a vasorelaxant agent that maintains vascular tone, decreases renal ischemia, mesangial cell proliferation and inflammatory cell infiltration (Yoon and Yang 2009). CsA administration in the present study significantly down-regulated the expression of renal eNOS mRNA but the overall effect on NO level had no change. This could be explained theoretically by accompanying the rise in iNOS production. Level of NO varies among different models in CsA nephrotoxicity. This difference is due to the balance between its different NOS isoforms (Shin et al., 2012; El-Kashef et al., 2016). The beneficial role of nicorandil in our model is thought to be mediated by NO/cGMP signaling pathway. This was evident by the significant elevation in NO level with eNOS up-regulation in CsA+nicorandil-treated rats. NO/cGMP was proved to inhibit the overexpression of inflammatory and fibrogenic mediators such as TGF-β and collagen proteins (Sudo et al., 2009; Tashiro et al., 2015).In accordance with others (Naesens et al., 2009; Mazali et al., 2012), administration of CsA was associated with increased TGF-β and collagen 1A1 mRNA expression in renal tissues. TGF-β, a growth factor released by macrophages, implicated in chronic CsA toxicity (Mazali et al., 2012) by promoting interstitial fibrosis and induction of various forms of renal injury (Ling et al., 2003). Treatment with nicorandil attenuated the overexpression of TGF-β and collagen 1A1 mRNA in CsA-intoxicated rats indicating its role as an anti-fibrotic agent. Our findings confirmed that apoptosis represents one of the mechanisms of renal injury induced by CsA results in tubular injury and loss of functional mass. Apoptosis is regulated by different caspases.Caspases mediate cell injury and anti-apoptotic agents responsible for cell survival (Yoon and Yang 2009). Caspase-3 that is specific for apoptosis was significantly increased in CsA group with a significant reduction in Bcl2 level, responsible for cell survival, which is in agreement with previous reports (Yang et al., 2002; Lai et al., 2015). Nicorandil treatment was able to protect tubular cells against CsA-induced apoptosis by regulation of apoptosis process. Immunostaining for caspase-3 was significantly attenuated in rats treated with nicorandil associated with significant increase in anti-apoptotic marker; Bcl2 level. In support of our results, other findings demonstrated that nicorandil inhibited apoptosis in different organs (Li et al., 2015; Zhang et al., 2015).

5. CONCLUSION

The present study demonstrated the promising defensive role of nicorandil against CsA-induced nephrotoxicity with different proposed mechanisms. Nicorandil exerts its protection through anti-oxidant role by attenuating lipid peroxidation and increasing anti-oxidant enzymes. In addition; significant anti-apoptotic and anti-fibrotic mechanisms were also explored.

Declaration of interest

The authors declare no conflict of interest.

6. REFERENCES

Ateyya, H., 2015. Amelioration of cyclosporine induced nephrotoxicity by dipeptidyl peptidase inhibitor vildagliptin. Int. Immunopharmacol 28, 571-577.

Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation.Methods.Enzymol 52, 302-310.

Chandramohan, Y., Parameswari, C.S., 2013. Therapeutic efficacy of naringin on cyclosporine (A) induced nephrotoxicity in rats: involvement of hemeoxygenase-1. Pharmacol. Rep 65, 1336-1344.

El-Kashef, D.H., El-Kenawi, A.E., Suddek, G.M., Salem, H.A., 2017. Allicin ameliorates kidney function and urinary bladder sensitivity in cyclosporine A-treated rats. Hum. Exp. Toxicol 36, 681-691.

Elshazly, S.M., 2015. Ameliorative effect of nicorandil on high fat diet induced non-alcoholic fatty liver disease in rats. Eur. J. Pharmacol 748, 123-132.

Fan, Y., Wei, Q., Cai, J., Shi, Y., Zhang, Y., Yao, L., Wang, X., Lin, S., Lv, J., Zhou, B., Du, R., 2016. Preventive effect of oral nicorandil on contrast-induced nephropathy in patients with renal insufficiency undergoing elective cardiac catheterization. Heart Vessels 31, 1776-1782.

Fawcett, J.K., Scott, J.E., 1960. A rapid and precise method for the determination of urea. J. Clin. Pathol 13,156-159.

Halloran, P.F., 1996. Molecular mechanisms of new immunosuppressants.Clin.Transplant 10,118-123.

Kim, J.H., Lee, Y.H., Lim, B.J., Jeong, H.J., Kim, P.K., Shin, J.I., 2016. Influence of cyclosporine A on glomerular growth and the effect of mizoribine and losartan on cyclosporine nephrotoxicity in young rats. Sci. Rep 6, 22374.

Korolczuk, A., Maciejewski, M., Smolen, A., Dudka, J., Czechowska, G., Widelska, I., 2014. The role of peroxisome-proliferator-activating receptor gamma agonists: rosiglitazone and 15-deoxy-delta12, 14-prostaglandin J2 in chronic experimental cyclosporine A-induced nephrotoxicity. J. Physiol. Pharmacol 65, 867-876.

Krajewska, M., Moss, S.F., Krajewskl, S., Song, K., Holt, P.R., Reed, J.C., 1996. Elevated expression of Bcl2 and reduced Bax in primary colorectal adenocarcinomas. Cancer Res 56, 2422-2427.

Lai, Q., Wei, J., Mahmoodurrahman, M., Zhang, C., Quan, S., Li, T., Yu, Y., 2015. Pharmacokinetic and nephroprotective benefits of using Schisandra chinensis extracts in a cyclosporine A-based immune-suppressive regime. Drug.Des.Devel.Ther 9, 4997-5018.

Li, W., Wu, N., Shu, W., Jia, D., Jia, P., 2015. Pharmacological preconditioning and postconditioning with nicorandil attenuates ischemia/reperfusion-induced myocardial necrosis and apoptosis in hypercholesterolemic rats. Exp. Ther. Med 10, 2197-2205.

Ling, H., Li, X., Jha, S., Wang, W., Karetskaya, L., Pratt, B., Ledbetter, S., 2003. Therapeutic role of TGF-beta-neutralizing antibody in mouse cyclosporin A nephropathy: morphologic improvement associated with functional preservation. J. Am. Soc. Nephrol 14, 377-388.

Marklund, S., Marklund, G., 1974. Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem 47, 469-474

Mazali, F.C., Johnson, R.J., Mazzali, M., 2012. Use of uric acid-lowering agents limits experimental cyclosporine nephropathy. Nephron.Exp. Nephrol 120, e12-19.

Moron, M.S., Depierre, J.W., Mannervik, B., 1979. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver.Biochim.Biophys. Acta 582, 67-78.

Naesens, M., Kuypers, D.R., Sarwal, M., 2009. Calcineurin inhibitor nephrotoxicity.Clin. J. Am. Soc. Nephrol 4, 481-508.

Ridnour, L.A., Sim, J.E., Hayward, M.A., Wink, D.A., Martin, S.M., Buettner, G.R., Spitz, D.R., 2000. A spectrophotometric method for the direct detection and quantitation of nitric oxide, nitrite, and nitrate in cell culture media.Anal. Biochem 281, 223-229.

Sanada, S., Node, K., Asanuma, H., Ogita, H., Takashima, S., Minamino, T., Asakura, M., Liao, Y., Ogai, A., Kim, J., Hori, M., Kitakaze, M., 2002. Opening of the adenosine triphosphate-sensitive potassium channel attenuates cardiac remodeling induced by long-term inhibition of nitric oxide synthesis: role of 70-kDa S6 kinase and extracellular signal-regulated kinase. J. Am. Coll. Cardiol 40, 991-997.

Schirmeister, J., Willmann, H., Kiefer, H., 1964. Plasma Creatinine as Rough Indicator of Renal Function. Dtsch. Med. Wochenschr 89,1018-1023.

Sereno, J., Rodrigues-Santos, P., Vala, H., Rocha-Pereira, P., Alves, R., Fernandes, J., Santos-Silva, A., Carvalho, E., Teixeira,F., Reis, F., 2014. Transition from cyclosporine-induced renal dysfunction to nephrotoxicity in an in vivo rat model. Intern.J. Mol. Sci 20, 8979-8997.

Shimizu, S., Saito, M., Kinoshita, Y., Ohmasa, F., Dimitriadis, F., Shomori, K., Hayashi, A., Satoh, K., 2011. Nicorandil ameliorates ischaemia-reperfusion injury in the rat kidney. Br. J. Pharmacol 163, 272-282.

Shin, B.C., Kwon, Y.E., Chung, J.H., Kim, H.L., 2012. The antiproteinuric effects of green tea extract on acute cyclosporine-induced nephrotoxicity in rats. Transplant. Proc 44, 1080-1092.

Shiraishi, T., Tamura, Y., Taniguchi, K., Higaki, M., Ueda, S., Shima, T., Nagura, M., Nakagawa, T., Johnson, R.J., Uchida, S., 2014. Combination of ACE inhibitor with nicorandil provides further protection in chronic kidney disease. Am. J. Physiol. Renal.Physiol 307, F1313-1322.

Singh, D., Chopra, K., 2004. The effect of naringin, abioflavonoid on ischemia-reperfusion induced renal injury in rats. Pharmacol. Res 50, 187-193.

Sudo, H., Hirata, M., Kanada, H., Yorozu, K., Tashiro, Y., Serizawa, K., Yogo, K., Kataoka, M., Moriguchi, Y., Ishizuka, N., 2009. Nicorandil improves glomerular injury in rats with mesangioproliferative glomerulonephritis via inhibition of proproliferative and profibrotic growth factors. J. Pharmacol. Sci 111, 53-59.

Takasu, C., Vaziri, N.D,, Li, S., Robles, L., Vo, K., Takasu, M., Pham, C., Liu, S., Farzaneh, S.H., Foster, C.E., Stamos, M.J., Ichii, H., 2015. Treatment With Dimethyl Fumarate Attenuates Calcineurin Inhibitor-induced Nephrotoxicity. Transplant 9, 1144-1150.

Tamura, Y., Tanabe, K., Kitagawa, W., Uchida, S., Schreiner, G.F., Johnson, R.J., Nakagawa, T., 2012. Nicorandil, a K (atp) channel opener, alleviates chronic renal injury by targeting podocytes and macrophages. Am. J. Physiol. Renal.Physiol 303, F339-349.

Tanabe, K., Lanaspa, M.A., Kitagawa, W., Rivard, C.J., Miyazaki, M., Klawitter, J., Schreiner, G.F., Saleem, M.A., Mathieson, P.W., Makino, H., Johnson, R.J., Nakagawa, T., 2012. Nicorandil as a novel therapy for advanced diabetic nephropathy in the eNOS-deficient mouse. Am. J. Physiol. Renal.Physiol 302, F1151-1160.

Tashiro, Y., Yogo, K., Serizawa, K., Endo, K., 2015. Nicorandil suppresses urinary protein excretion and activates eNOS in Dahl salt-sensitive hypertensive rats. Clin. Exp. Nephrol 19, 343-349.

Yang, C.W., Faulkner, G.R., Wahba, I.M., Christianson, T.A., Bagby, G.C., Jin, D.C., Abboud, H.E., Andoh, T.F., Bennett, W.M., 2002. Expression of apoptosis-related genes in chronic cyclosporine nephrotoxicity in mice.Am. J. Transplant 2, 391-39.

Yong-Gang, X., Ming-Zhe, W., Jin-Yan, Z., Zhi-Hai, P., Jun-Ming, X., 2014. Combination of N-(3'4'-dimethoxycinnamoyl) anthranilic acid with cyclosporin A treatment preserves immunosuppressive effect and reduces the side effect of cyclosporin A in rat. Eur. J. Pharmacol 728, 16-23.

Yoon, H.E., Yang, C.W., 2009. Established and newly proposed mechanisms of chronic cyclosporine nephropathy. Korean J. Intern. Med 24, 81-92.

Zhang, F., Cui, J., Lv, B., Yu, B., 2015. Nicorandil protects mesenchymal stem cells against hypoxia and serum deprivation-induced apoptosis. Int. J. Mol. Med 36, 415-423.

Zhang, H.Y., Meng, X., Du, Z.X., Fang, C.Q., Liu, G.L., Wang, H.Q., Deng, W.W., 2009. Significance of survivin, caspase-3, and VEGF expression in thyroid carcinoma.Clin. Exp. Med 9, 207-213.