Hesperidin protects gentamicin-induced nephrotoxicity via Nrf2/HO-1 signaling and inhibits inflammation mediated by NF-κB in rats

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ABSTRACT
The defensive effects of hesperidin (HDN, a bioflavonoid) was investigated on gentamicin (GEN) provoked nephrotoxicity in rats. The expression patterns of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), nuclear factor-κB (NF-κB), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), kidney injury molecule (KIM-1), osteopontin, heme oxygenase-1 (HO-1), inducible nitric oxide synthase (iNOS) and heat shock protein-70 (HSP-70) were assessed to comprehend the mechanism of action of hesperidin. GEN treated rats showed increased expressions of KIM-1, osteopontin, COX-2, Nrf2, NF-κB, TNF-α, iNOS, IL-6 and HSP-70 and decreased expression of HO-1. HDN along with GEN decreased the expressions of all indices excluding HO-1 and Nrf2 and an inverse correlation of expression was seen in between Nrf2 and NF-κB. Scavenging of free radicals, suppression of inflammation, upregulation of HO-1 by Nrf2 mediated antioxidant response element system (ARE) and facilitation of antioxidative system by HDN would be responsible to thwart GEN induced renal damage.

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1. Introduction
The aminoglycoside antibiotic gentamicin (GEN) is extensively used in clinical practice for the management of acute Gram-negative bacterial diseases (Valle, Imbrogno, & Fernandez, 1969). Nonetheless, a main impediment in the utilization of aminoglycoside antibiotics is their potent nephrotoxicity (Martinez-Salgado, Lopez-Hernandez, & Lopez-Novoa, 2007). Gentamicin is renowned to cause acute renal failure among

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Abbreviations: ARE, antioxidant response element; CAT, catalase; COX-2, cyclooxygenase-2; ECH, echinoderm microtubule associated; ERK, extracellular signal-regulated kinases; GEN, gentamicin; GST, glutathione-S-transferase; HDN, hesperidin; HO-1, haem oxygenase 1; HRP, horseradish peroxidase; HSP-70, heat shock protein-70; IL-6, interleukin-6; iNOS, inducible nitric oxide synthase; KIM-1, kidney injury molecule 1; MAPK, mitogen-activated protein kinases; NF-κB, nuclear factor-xB; NO, nitric oxide; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PBS, phosphate buffered saline; ROS, reactive oxygen species; RT-PCR, reverse transcriptase-polymerase chain reaction; SOD, superoxide dismutase; TBS, Tris buffered saline; TNF-α, tumor necrosis factor-α.
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Moreira et al., 2014. GEN has been documented to enhance the generation of ROS (reactive oxygen species) (Moreira et al., 2014). Elevated production of ROS induces cellular injury and necrosis through peroxidation of membrane lipids, protein denaturation and DNA damage. The kidneys from GEN-treated rats are more susceptible to ROS damage owing to the insufficiency of antioxidant enzymes (Com et al., 2012; Khan et al., 2009). GEN induces superoxide anion (O2•−), hydrogen peroxide (H2O2), and hydroxyl radical (•OH) production from renal mitochondria (Moreira et al., 2014). In addition, H2O2 generation (Moreira et al., 2014), lipid peroxidation, nitrotyrosine, protein oxidation (Sener et al., 2002) and protein carbonyl (Kovacs et al., 2012) production mediate nephrotoxic effects of GEN. Furthermore, reduced glutathione content is diminished (Sener et al., 2002) in renal cortex of GEN-treated rats. GEN was shown to induce apoptosis in renal cortical cells in rats (Hsu et al., 2014). Earlier studies explored that GEN could release iron from renal mitochondria and form an iron–GEN complex which enhances the production of free radicals and ROS (Kovacs et al., 2012).

The antioxidant elevating activity, histoprotective activity of renal tissue, and normalization of kidney myeloperoxidase (MPO) and urinary enzymes (β-N-acetyl glucosaminidase (NAG), β-glucuronidase and γ-glutamyl transferase (γ-GTP)) was previously reported upon hesperidin administration in GEN-treated rats (Anandan & Subramanian, 2012). Hesperidin (HDN, 3, 5, 7-trihydroxyflavanone 7-rhamnoglucoside) is an abundant bioflavonoid present in citrus fruits, sweet oranges, tangelos and lemon (Garg, Garg, Zaneveld, & Singla, 2001; Russo, Bonaccorsi, Inferregra, Dugo, & Mondello, 2015). HDN has been shown to exhibit a variety of pharmacological effects including antioxidant, free radical scavenging, anti-inflammatory, anti-allergenic, neuroprotective, antihypertensive, antimicrobial, hypolipidemic, anticarcinogenic, vasodilatory, analgesic and antihypercholesterolemic properties (Abuelsaad, Mohamed, Allam, & Al-Solumani, 2013; Chen et al., 2013; Kamaraj, Ramakrishnan, Anandakumar, Jagan, & Devaki, 2009; Kaur, Tirkey, & Chopra, 2006; Rong et al., 2013; Sahu, Kuncha, Sindhura, & Sista, 2013; Tirkey, Pilkhalw, Kuhad, & Chopra, 2005). HDN demonstrates antioxidative properties by diverse mechanisms, including scavenging of free radicals, prevention of DNA damages and chelation of metal (iron and copper) ions (which play key roles in the initiation radical reactions), repression of enzymes accountable for free radical generation and enhancement of endogenous antioxidative defense system (Cao, Sofic, & Prior, 1997; Chen et al., 2010; Oliveira et al., 2013; Sahu et al., 2013).

Despite identification of GEN nephrotoxicity and intensive scientific attempts directed into developing therapeutic or prophylactic agents to protect against GEN nephrotoxicity, conventional therapeutic options accessible to either treat or prevent its adverse effects, are inadequate. Although GEN-induced nephrotoxicity appears to be directly reconnected to its concentrated accrual in the renal proximal tubular cells (Martinez-Salgado et al., 2007), the mechanisms involved in GEN induced cell injury are not completely understood. In the present study, the nephroprotective effects of hesperidin by assessing transduction factors, inflammatory mediators, renal markers, a cytokine and a phase II enzyme (kidney injury molecule (KIM-1), osteopontin, haem oxygenase (HO-1), cyclooxygenase-2 (COX-2), nuclear factor (erythroid-derived 2)-like 2 (Nrf2), nuclear factor-κB (NF-κB), tumor necrosis factor-α (TNF-α), inducible nitric oxide synthase (iNOS), interleukin-6 (IL-6) and heat shock protein-70 (HSP-70)) were assessed in order to comprehend the mechanism of action of hesperidin on GEN-induced nephrotoxicity.

KIM-1, a tubular protein, is markedly induced in response to a number of nephrotoxins (Ichimura, Hung, Yang, Stevens, & Bonventre, 2004) and has been considered as a sensitive marker of proximal tubule injury (Jin et al., 2013). Osteopontin is known to express in renal tubular cells under various experimental or pathological conditions in vivo or in vitro (Chen et al., 2009; Jin et al., 2013), experimental cyclosporine nephropathy (Pichler et al., 1995) and GEN-provoked nephrotoxicity (Com et al., 2012). HO-1, a protective gene in kidney, is entailed in the production of anti-inflammatory, antioxidant, and antiapoptotic metabolites (He et al., 2014). Phase II genes, including HO-1, SOD, CAT, GST and NAD(P)H quinine oxidoreductase, function in synergy to scavange ROS or reactive nitrogen species (RNS), maintain intracellular reducing potential, and detoxify xenobiotics and electrophiles (Sathoh et al., 2006). The transcription factor, Nrf2 regulates ARE (antioxidant response element)-driven HO-1 gene expression (Zhang et al., 2013). COX-2 plays key role in prostaglandin biosynthesis and inflammation and is expressed constitutively in kidney (Rios, Vargas-Robles, Gamez-Mendez, & Escalante, 2012). The 5′-flanking region of the COX-2 promoter contains NF-κB binding sites and a major molecule subjected to NF-κB-driven transactivation is COX-2 (Chun & Surh, 2004).

The two redox-sensitive transcription factors, that determine the role of transcriptional adaption to chemical stress, are Nrf2 and NF-κB. Nrf2, the chief regulator of the antioxidant response (Nguyen, Nioi, & Pickett, 2009) initiates the response by binding to the ARE in the promoter region of numerous genes, encoding for antioxidative and phase II enzymes in various types of cells (Cheng, Kalabuz, Zhang, & Blanco, 2012). NF-κB (transcription factor) activation is regulated by intra-/extra-cellular ROS and/or ROS-modified target biomolecules (Gonzalez-Ramos, Defrere, & Devoto, 2012). The expression of genes which are involved in inflammation, cell proliferation and cytokine production is mainly regulated by NF-κB (Gonzalez-Ramos et al., 2012). Upon stimulation, NF-κB is released from its inhibitory subunit (IkB) and translocates into the nucleus, where it enhances the transcriptional activation of target genes, for instance, IL-6, iNOS, TNF-α and COX-2 (Amigo, Paya, Braza-Boils, De Rosa, & Terencio, 2008; Gonzalez-Ramos et al., 2012; Ozbek et al., 2009).

TNF-α is an effective proinflammatory cytokine, produced by renal mesangial and epithelial cells which could influence progression of diseases and enhance the production of other cytokines interceding inflammation (Benedetti et al., 2013). GEN is known to increase macrophage infiltration and elevate TNF-α level that led to the progression of nephritis (Sahu et al., 2013). Nitric oxide (NO) is produced from a wide variety of cell types by nitric oxide synthase enzymes (NOS) and surfeit NO could elevate RNS (Sunil et al., 2012). A type of NOS, iNOS (inducible NOS), typically entails the presence of some inducing agent (e.g., cytokines) for expression, consequently forms large amounts of NO (Csont et al., 2005). IL-6, a pro-inflammatory cytokine is primarily involved in the regulation of immune and
inflammatory responses (Fielding et al., 2008). Heat shock proteins (HSPs) are induced in cells in response to shock, hyperthermia, and other environmental stresses (Kalmar & Greensmith, 2009). Major HSPs are known to localize in renal tubular epithelial cells and GEN specifically binds to C-terminal region of HSP-70 (Yamamoto et al., 2010).

Our earlier study (Anandan & Subramanian, 2012) showed that rats treated with GEN exhibit desquamation, degeneration, and vacuolization of proximal tubules, increased number of hyaline casts in renal tubules, marginal localization of chromatin in the nuclei, mononuclear cell infiltration, intertubular hemorrhage, picnosis, atrophied glomeruli, and obliteratorative arteriolaropathy in renal tissues, including inflammatory responses (Anandan & Subramanian, 2012). In rats administered with HDN and GEN, the renal tubules were normal with mild mononuclear cell infiltration, slight degenerative epithelial changes in glomeruli and tubes, and a reduction in sporadic hyaline casts in tubes (Anandan & Subramanian, 2012). We performed a mechanistic approach for the effect of HDN via Nrf2/HO-1 signaling and scrutinized whether the inhibition of inflammation is mediated by NF-κB in this study.

2. Materials and methods

2.1. Experimental animals

All the experiments were performed in male rats (Wistar strain, 180–200 g), acquired from the Central Animal House, Faculty of Medicine, Annamalai University. The animals were housed (4 rats per cage) and maintained on 12:12 h day and night cycles at 25 ± 2 °C. The animals were fed with standard pellet diets and drinking water ad libitum. The experiments involved with animals were accomplished according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), New Delhi, India and endorsed by the Animal Ethical Committee of Annamalai University (Approval No.160/1999 CPCSEA).

2.2. Chemicals and antibodies

Gentamicin sulfate (GEN) was purchased from Ranbaxy Laboratories Limited, New Delhi, India. HDN, thiobarbituric acid, phenazine methosulfate, nitroblue tetrazolium, adenosine triphosphate and nicotinamide adenine dinucleotide were purchased from Sigma Chemical Company, St. Louis, MO, USA. Total RNA isolation kit and primers for PCR were obtained from Bangalore Genei, Bangalore, India. The RT-PCR kit was procured from Qualigen, Carlsbad, CA, USA. All other chemicals and biochemicals used in the study were obtained from S.D. Fine Chemicals Ltd., Mumbai, India and were of analytical grade.

The primary antibodies (polyclonal anti-rat Nrf2, NF-κB p65, TNF-α, iNOS, IL-6 and HSP-70) were acquired from Santa Cruz Biotech, Dallas, TX, USA. Secondary antibodies (HRP-conjugated anti-rabbit IgG for Western blotting) were purchased from Bangalore Genei.

2.3. Induction of nephrotoxicity and tissue homogenate preparation

HDN in saline (0.9% NaCl) was administered orally at dose of 200 mg/kg body weight (Anandan & Subramanian, 2012; Tirkey et al., 2005). GEN (100 mg/kg body weight) was injected to animals intraperitoneally (Anandan & Subramanian, 2012; Parlakpinar et al., 2005). GEN injections were carried out between 09.00 and 09.30 h to maximize GEN-provoked nephrotoxicity as circadian variation in GEN-induced nephrotoxicity is well known (Pariat, Courtous, Cambar, Piriou, & Bouquet, 1988).

After an acclimatization period of 7 days, rats were stratified by body weight and allocated into following 4 groups (n = 8/group). Adequate sets of the following groups were maintained for RT-PCR, Western blotting and immunohistochemical studies.

Group I: control rats.

Group II: rats injected with GEN (100 mg/kg b.w.) for last eight days (15th to 22nd day).

Group III: rats received HDN (200 mg/kg b.w.) orally for the entire experimental period (22 days) and GEN (100 mg/kg body weight) injected from 15 to 22 days.

Group IV: rats given with HDN (200 mg/kg b.w.) orally for 22 days.

At the end of experimental period, all animals were sacrificed by cervical dislocation. The kidney tissues were excised, sliced into pieces (250 mg) and homogenized in appropriate buffer in cold condition (pH 7.0) to give 20% homogenate (w/v). The homogenate was centrifuged at 1000 rpm for 10 min at 4 °C. The supernatant was used for various analyses.

2.4. RT-PCR

Total RNA was isolated from the tissues using RNA isolation reagent (Trizol) (Chomczynski & Sacchi, 1987) and RT-PCR was performed by using the appropriate primer pairs (Table 1). Total reaction of 25 μL was performed in 250 μL PCR vial. Amplification reaction mixture contains 2.5 μL of cDNA, 200 μM of the four dNTPs, 100 pmol of each primer, and 2.5 U of Taq DNA polymerase (Bangalore Genei). The samples were incubated in the thermocycler as per the following reactions: initial denaturation (94 °C, 2 min), denaturation (94 °C, 30 s), annealing (temperature varies depending on gene, 30 s), extension (72 °C, 4 min). After each step, the

<table>
<thead>
<tr>
<th>Gen product</th>
<th>Primer sequence</th>
<th>Fragment size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIM-1</td>
<td>Forward-5′ACTCCTGCAAGCTGCAATG3′</td>
<td>213</td>
</tr>
<tr>
<td>Osteopontin</td>
<td>Reverse-5′CAAAGGTCAAGGCCCCTG3′</td>
<td>416</td>
</tr>
<tr>
<td>HO-1</td>
<td>Reverse-5′CAGAAAGGCTCAGGTTTGC3′</td>
<td>260</td>
</tr>
<tr>
<td>COX-2</td>
<td>Forward-5′CTGAAGGTTGTTACACTTCG3′</td>
<td>209</td>
</tr>
<tr>
<td>β-actin</td>
<td>Reverse-5′TGACAAAAATGTGTTACG3′</td>
<td>253</td>
</tr>
<tr>
<td>18S RNA</td>
<td>Forward-5′TTCCCTTTTGTGTCGGCCTCC3′</td>
<td>510</td>
</tr>
</tbody>
</table>

Table 1 – Oligonucleotide primers used for RT-PCR.
contents of the vial were mixed gently and centrifuged briefly. RT-PCR was performed with 18S RNA and β-actin as internal standards. PCR products (5 μl) were separated on a 2% agarose gel and the images were photographed using Gel Doc XR system (Bio-Rad, Hercules, CA, USA) while intensities of the bands were quantified using Quantity on 1-D analysis software and plotted (Fig. 1).

2.5. Western blotting

Fifty micrograms of total protein was added with 2X sample buffer and boiled for 5 min. The sample mixture was run on 12% SDS-PAGE gel and electrottransferred to a PVDF membrane (Millipore, Schwalbach, Germany). The membrane was blocked in blocking buffer containing 5% skimmed milk powder for 2 h. After 2 h, the blocked membranes were incubated with primary antibody (rat polyclonal Nrf2 and NF-κB p65) diluted (1:1000) with blocking buffer for 6 h. Subsequent to primary antibody incubation, the membranes were washed three times with blocking buffer for 10 min each. Membranes were incubated with secondary antibody (anti-rat IgG alkaline phosphatase conjugate, Bangalore Genei Pvt. Ltd.). Specific binding was identified using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) as substrates with β-actin as internal control. Membrane with bands was scanned using a scanner (HP Scan) and the intensity of bands was quantified using the Biorad Quantity One 1D Analysis Software (BioRad) and were plotted (Fig. 2).

2.6. Immunohistochemical analysis

The glass section slides were dewaxed and rehydrated through a steady decrease in ethanol concentration. The slides were incubated in sodium citrate buffer (pH 6.0) for two cycles of 5 min in a microwave oven for antigen retrieval. After washing with PBS, the slides with tissue section were treated with hydrogen peroxide (3%) to remove any endogenous peroxidases. Blocking was carried out at 4 °C using normal goat serum in PBS, the slides with tissue section were treated with hydrogen peroxide (3%) to remove any endogenous peroxidases. Blocking was carried out at 4 °C using normal goat serum in PBS. After washing with PBS, the slides with tissue section were treated with hydrogen peroxide (3%) to remove any endogenous peroxidases. Blocking was carried out at 4 °C using normal goat serum in PBS. The glass section slides were dewaxed and rehydrated through a steady decrease in ethanol concentration. The slides were incubated in sodium citrate buffer (pH 6.0) for two cycles of 5 min in a microwave oven for antigen retrieval. After washing and PBS, the slides with tissue section were treated with hydrogen peroxide (3%) to remove any endogenous peroxidases. Blocking was carried out at 4 °C using normal goat serum in PBS. After washing with PBS, the slides with tissue section were treated with hydrogen peroxide (3%) to remove any endogenous peroxidases. Blocking was carried out at 4 °C using normal goat serum in PBS.

2.7. Statistical analysis

The data for various indices were analyzed using analysis of variance (ANOVA) and the group means were compared by Duncan’s Multiple Range Test (DMRT). Values were considered statistically significant when p < 0.05.

3. Results

3.1. RT-PCR analysis of KIM-1, osteopontin, HO-1 and COX-2

KIM-1 and osteopontin expressions were found to be significantly increased (p < 0.05) in the kidney of GEN induced rats (compared to controls) whereas in GEN + HDN treated rats they were appreciably decreased (p < 0.05; Fig. 1). In HDN treated rats, there was no significant variation of expression of KIM-1 and osteopontin compared to control (Fig. 1). GEN induced rats displayed a decreased expression of HO-1 when compared to controls (Fig. 1). HDN treatment to GEN-induced group elevated the expression level of HO-1. HDN treated group exhibited insignificant variation compared to control group. RT-PCR analysis of COX-2 showed its upregulated expression in GEN-provoked nephrotic animals. COX-2 expression was considerably decreased in HDN pre-treated animals as compared with GEN-treated animals (Fig. 1).

3.2. Expression of Nrf2 and NF-κB p65 by western blotting

Western blot analysis showed detectable levels of Nrf2 in control group (Fig. 2). GEN treatment increased the expression of Nrf2. HDN treatment to GEN-induced group resulted in further elevation in the expression of Nrf2 (Fig. 2). Furthermore, the expression of Nrf2 is significantly elevated (p < 0.05) in HDN treated group (compared to control) (Fig. 2). The expression of NF-κB p65 in the kidney is increased (p < 0.05) after GEN treatment (Fig. 2). In contrast, HDN treatment decreased the expression of NF-κB p65 significantly (p < 0.05) in GEN-treated rats. The expression in HDN treated group is insignificantly different compared to control group (Fig. 2).

3.3. Immunohistochemical expression of TNF-α, iNOS, IL-6 and HSP-70

Control rats showed the basal level of immunohistochemical staining of TNF-α, iNOS, IL-6 and HSP-70 in kidney tissues (Fig. 3). The expression levels of TNF-α, iNOS, IL-6 and HSP-70 are elevated in kidney tissues of GEN-treated rats. Conversely, HDN + GEN treatment showed considerable reduction of these expressions as compared to GEN-induced rats (Fig. 3). The HDN treated rats showed similar pattern of expressions of TNF-α, iNOS, IL-6 and HSP-70 to that of controls (Fig. 3).

4. Discussion

The specificity of GEN-provoked renal toxicity is related to its preferential accrual in the renal convoluted tubules, lysosomes, brush border membranes and basolateral membranes by binding with the phosphatidyl-inositide receptors (Nagai, Saito, Adachi, Yumoto, & Takano, 2006; Zeeni, Selmaoui, Beauchamp, Labrecque, & Thibault, 2007). This aminoglycoside further inhibits phospholipases and sphingomyelinase activity causing the production of myeloid bodies. The development...
Fig. 1 - Representative images of agarose gel electrophoresis (i) A, (ii) A, (iii) A and (iv) A showing RT-PCR products of KIM-1, osteopontin, HO-1 and COX-2 respectively. In (i) lanes M, 1, 2, 3 and 4 represent molecular weight marker, control, GEN, GEN+HDN and HDN treated groups respectively (β-actin expression); lanes 5, 6, 7 and 8 denote control, GEN, GEN+HDN and HDN treated groups respectively. β-actin (in (ii)) and 18S RNA (in (iii) and (iv)) were used as controls. The intensity of the bands was quantified by densitometric analysis and expressed in relative intensity arbitrary units in panels (i) B, (ii) B, (iii) B and (iv) B for KIM-1, osteopontin, HO-1 and COX-2 correspondingly. Each value represents the mean ± SD for 8 rats. Data points with different superscripts are significantly different at the level of $p < 0.05$ by DMRT.
of these tightly placed lipidic bilayers results in swelling of lysosomes and release of excess amounts of accumulated aminoglycoside, lysosomal enzymes and phospholipids into the cytosol. Necrosis of proximal tubular cells pursues and lead to impairment of renal function (Nagai et al., 2006; Zeeni et al., 2007). Flavonoids containing hydroxyl groups, particularly those having an O-dihydroxy group on ring B, like HDN, appear to be effective free radical scavengers (Garg et al., 2001). Flavonoids can inhibit free radical formation and the dissemination of free radical reactions through the chelation of metal ions (Andjelković et al., 2006). The free radical scavenging activity of HDN could be owing to the presence of 3′-hydroxy, and 4′-methoxy group on aromatic ring (B), which provides hydrogen and an electron to counteract the hydroxyl and superoxide free radicals (Cao et al., 1997). In addition to the chelation of metal ions, HDN inhibits the superoxide-derived Fenton reaction, which is a chief source of reactive hydroxyl radicals (Mladenka et al., 2011).

Elevations in the expression of KIM-1, clusterin, lipocalin 2, osteopontin and kallikrein have been reported in various models of renal injury (Ichimura et al., 2004; Yoshida et al., 2002). KIM-1 is a reliable, sensitive and early biomarker of renal diseases entailing acute injury of the proximal tubule epithelium (Jin et al., 2013) and it is known to co-express with renal osteopontin, and collagen III (Huo, Zhang, Nie, Li, & Jin, 2010). Our results demonstrated elevated expression of KIM-1 in GEN-induced rats and it could be mainly owing to tubulointerstitial damage. However, co-administration of HDN distinctly suppressed KIM-1 expression in kidney and it might be due to its protective effect on the renal tubular cells. Osteopontin has also been proposed as a potential marker of kidney toxicity (Amin et al., 2004) and investigations have suggested that increased osteopontin mRNA expression might be associated with monocyte or macrophage infiltration in renal tubules (Khan et al., 2009). The significant elevation of osteopontin expression in kidney tissues in GEN treated rats in our study is in parallel with previous findings (Khan et al., 2009). HDN treatment restored osteopontin expression to normal which might be attributed to its immunomodulatory, cytoprotective and anti-inflammatory properties. Earlier investigations have shown that Nrf2 could play a major role in regulating HO-1 expression (Chen et al., 2009; Cheng et al., 2012). HO-1 is a stress-responsive phase II enzyme, extensively distributed in many mammalian tissues, which is responsible for the breakdown of haem (Maines & Gibbs, 2005) and breakdown of haem compounds would eventually neutralize the oxidative stress imposed by ROS (He et al., 2014; Maines & Gibbs, 2005). In our study, HDN was shown to upregulate the HO-1 expression, which suggested that HDN might induce phase II genes through the ARE system. HDN facilitated MAPK/ERK phosphorylation and could be accountable for nuclear translocation of Nrf2, followed by cytoprotective HO-1 expression (Chen et al., 2010). Furthermore, HDN could also enhance the antioxidant defense through the induction of HO-1, as observed in our study, probably via ERK/Nrf2 signaling (Chen et al., 2010) and thereby acts as a potential therapeutic agent in preventing renal injury. Activated NF-κB regulates the transcription of COX-2, which is entailed in prostaglandin biosynthesis and inflammation (Chun & Surh, 2004). Our RT-PCR results of COX-2 revealed its upregulation at the transcriptional level in GEN-induced animals suggesting the involvement of inflammation. Pretreatment with HDN attenuated the inflammation probably via COX-2. Multiple lines of
Evidence suggest that flavonoids could possess anti-inflammatory effect by targeted inhibition of NF-κB mediated expression of COX-2 enzyme (Chun & Surh, 2004) corroborating our results.

Evidence emphasized that Nrf2 has emerged as a crucial regulator of both constitutive and inducible expression of cytoprotective (phase II) genes in diverse cell types (Copple, Goldring, Kitteringham, & Park, 2008). In particular, Nrf2 is expressed in tissues related with detoxification such as liver and kidney and those that are rendered to the external environment (Copple et al., 2008). Electrophiles and ROS could release Nrf2 from its cytoplasmic Keap1, incite the translocation and accrual of Nrf2 in the nucleus (Itoh et al., 1999; Niture, Kaspar, Shen, & Jaiswal, 2010). In the present investigation, HDN with GEN treated animals exhibited a higher level of Nrf2 expression when compared to GEN alone treated group. This might be possible owing to the effect of ROS or electrophiles provoked by GEN and the ability of HDN to stimulate Nrf2 pathway. The plausible mechanism for HDN’s action may occur through the phosphorylation of cystine thiols in Keap1 protein and/or

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**Fig. 3 – Immunohistochemical staining of TNF-α, iNOS, IL-6 and HSP-70 are represented in panels (i), (ii), (iii) and (iv) respectively. Photomicrographs A, B, C and D represent control, GEN, GEN + HDN and HDN treated groups correspondingly. Minimal positive signals in (i) A, (ii) A, (iii) A and (iv) A, increased expression patterns in (i) B, (ii) B, (iii) B and (iv) B, marked reduction of signals in (i) C, (ii) C, (iii) C and (iv) C and near normal signals in (i) D, (ii) D, (iii) D and (iv) D are seen.**
of serine and threonine residues in Nrf2 by protein kinases consequently leading to the dissociation of Keap1-Nrf2 complex for the expression of phase II detoxifying enzymes (Chen et al., 2010). However, the mechanism by which HDN exhibits induction of antioxidants and phase II genes via Nrf2/HO-1 signaling in GEN-treated rats still remains unclear. Phase II gene inducers react more avidly with Keap1 than with Nrf2 (Dinkova-Kostova et al., 2002). HDN, as shown in this study could stimulate Nrf2 translocation, plausibly by dissociating the Nrf2–Keap1 complex (Fig. 4). The transcription factor NF-κB is activated by a wide range of chemical and biological stimuli including ROS (Amigo et al., 2008). Upon stimulation, NF-κB dissociates from the complex (NF-κB–IκB), translocates into the nucleus and subsequently (Fig. 4) enhances the transcriptional activation of target genes (Amigo et al., 2008; Lawrence, Gilroy, Colville-Nash, & Willoughby, 2001) including IL-6, iNOS, TNF-α, COX-2, that play crucial roles in preventing cellular damage, oxidative stress mediated reactions and inflammation (Lawrence et al., 2001). The increased expression of NF-κB in GEN treated rats could be attributed to increased oxidative stress and elevated NF-κB could in turn induce the synthesis of other inflammatory related molecules (cytokines, growth factors and adhesion molecules) that enhance kidney damage. The anti-inflammatory effect of HDN might be associated with diminished NF-κB expression in GEN-induced nephrotoxicity (Nagashio et al., 2013). Disruption of Nrf2 was found to enhance the up-regulation of NF-κB and pro-inflammatory cytokines in the brain of rats after traumatic brain injury (Jin et al., 2008; Mao et al., 2010). The present results revealed the possibility that Nrf2-mediated nephroprotective effect may be achieved by the suppression of NF-κB (Fig. 4).

Cytokines that are induced by NF-κB, particularly, TNF-α and IL-6, can also directly activate the NF-κB pathway, thus establishing a positive auto regulatory loop that can augment the inflammatory response and duration of inflammation (Beg & Baltimore, 1996). TNF-α and IL-6 are the most multifaceted cytokines known to regulate inflammation, abnormal cell growth, and immunomodulation. Thus, blockade of TNF-α and IL-6 will be a valuable approach (Sahu et al., 2013) for the management of nephrotoxicity. Intriguingly, in our study, HDN possesses anti-inflammatory property and decreased TNF-α and IL-6 expressions. Yamamoto, Suzuki, Jokura, Yamamoto, and Tase (2008) demonstrated the blood pressure lowering effect of HDN through nitric oxide-mediated vasodilation in rats. Numerous oxidative stressors can induce the co-expression of inducible iNOS and COX-2, which synthesize NO and prostaglandin, respectively (Rios et al., 2012). The elevated expression of iNOS is known to be accountable for the overproduction of NO associated with inflammation in GEN-induced nephrotoxicity (Ghaznavi & Kadkhodaei, 2007). Among diverse radicals involved in damage of kidney cells, iNOS appears to play a main role, and that the blockade of iNOS could diminish GEN-provoked nephrotoxicity effectively (Ghaznavi & Kadkhodaei, 2007). Reduction of both oxidative stress and iNOS expression may be responsible for the protective effect of HDN on GEN-provoked structural and functional alteration of kidney. Studies have shown that HSP-70 can be induced in renal cells
during acute renal failure due to ischemia and nephrotoxic drugs, and suggested their cytoprotective roles (Saito et al., 2004; Tolson, Roberts, Jortner, Pomeroy, & Barber, 2005). The present immunohistochemical study revealed the increased expression of HSP-70 in renal tissues owing to nephrotoxicity and HDN could effectively suppress the HSP-70 probably through its antioxidant and cytoprotective actions.

To summarize, HDN supplementation inhibited NF-κB mediated expression of inflammatory genes (IL-6, iNOS, TNF-α, COX-2) and upregulated Nrf2 and Nrf2 dependant phase II antioxidant gene HO-1. The effects of HDN might be attributed to multiple mechanisms such as scavenging of free radicals, suppression of inflammation, chelation of metal ions, inhibition of enzymes responsible for free radical generation and facilitation of endogenous antioxidative defense system. Based on the results, HDN could be a potential therapeutic agent in the treatment of oxidative stress-related renal cell injury and renal dysfunction. The potential therapeutic action of HDN to protect or reverse renal GEN damage would have indispensable clinical consequences.

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REFERENCES


