Hepato & nephro protective effects of naringenin-loaded tpgs polymeric nanosuspension against cisplatin-induced toxicity

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ABSTRACT
Cisplatin (Cis-Diammineplatinum (II) dichloride/CIS) is one of the most potent chemotherapeutic agents widely used in treatment of various cancers. Naringenin (NAR), a natural flavonoid, protect against CIS-induced injury in rats without hampering CIS beneficial cytotoxic activity. Even though NAR exhibits therapeutic potency, clinical evolution of the molecule is embarrassed because of very less aqueous solubility which corresponds to low availability at the site of the tumor. In our former analysis, nanosuspension of naringenin (NARNS) was developed by the method of high-pressure homogenization. The study had been continued to evaluate the protective role of D-α-Tocopheryl polyethylene glycol succinate (TPGS) coated NARNS, against oxidative stress-induced hepato and nephrotoxicity in male Wistar rats upon CIS treatment. Induction of acute hepato and nephrotoxicity was done by intraperitoneal injection (i.p) injection of CIS (7 mg/kg of body weight) and administration of NAR and NARNS. Administration of NARNS virtually suppressed CIS-induced and liver injury evidenced by a reduction of lipid peroxidation level, blood urea nitrogen, serum uric acid, creatinine and elevated enzymatic antioxidant activities of superoxide dismutase, catalase, and glutathione peroxidase in rats liver tissue. Histological studies substantiated the biochemical parameters. The study suggests that NARNS has strong hepato and nephroprotective effect compared to NAR.

INTRODUCTION

Even though CIS platin is an extensively used chemotherapeutic agent, it uses causes a wide range of side effects since they effect normal and cancerous tissues. Ototoxicity, neurotoxicity, nephrotoxicity, and hepatotoxicity are the major adverse events occurs due to the drug administration. CIS induced cytotoxicity is a contribution to reduce its application in therapy. Reactive oxygen species generation in cells caused by the CIS oxidative harm to mitochondria and inhibition of the antioxidant enzymes and arresting their activity leads to the release of free radicals. These mechanisms result in tissue damage and the toxic effect caused by CIS. Naringenin a flavonoid with its proved protec-
ative mechanism in inflammation, antioxidant, neuroprotective, immunomodulator, anticarcinogenic agent, antiatherogenic and antiapoptotic activity were proved by the previous studies. Naringenin nanosuspension was formulated to overcome the bioavailability and solubility problems associated with the drug and studied further for their anti-inflammatory activity. This formulation with a significantly high anti-inflammatory activity compared to naringenin was studied further in the current study to evaluate the hepatoprotective and nephroprotective activity against CIS platin induced toxicity (Yen et al., 2009; Sumathi et al., 2017).

MATERIALS AND METHODS

Chemicals

TPGS was procured from commercial supplier Ludwigshafen, Germany. Cis was purchased from Sigma-Aldrich, Germany. NAR was bought from Zim laboratories Limited, Nagpur. Soya lecithin was obtained from Glenmark generics limited, Mumbai, India. And Chemicals purchased and used were of analytical grade.

Animals

Male albino Wistar rats weighing 130-140g were purchased for the study. Animals were exposed to controlled environmental conditions with constant temperature (25 ± 2 °C) and humidity (55 ± 5%) and fed with laboratory feed and water. They were under a dark/light cycle of 14/10 h. Animals were in the specified laboratory conditions prior a week from the start of the study to ensure the animals were acclimatized to it. Institutional animal ethics committee (NCP/IAEC/2015-2016-04) was there for the performance of the study.

Hepatoprotective activity

30 Albino Wistar strain rats were equally distributed to five groups, each containing six animals. Intraperitoneal injection of (7 mg/kg b.w.) was administered to induce CIS-toxicity. Group I(Control group): Animals were considered as normal control throughout the study procedure. Group II was treated with a single dose of cisplatin i.p on the fifth day of the experiment at a dose of 7 mg/kg (Koyuncu et al., 2017). Group III, rats of this group were administered with silymarin (10 mg/kg i.p; once daily) for 9 days, and on 5th day CIS (7 mg/kg b w) was injected by i.p route (Singh et al., 2014). Group IV (CIS-NAR) rats of this group were administered with NAR (50 mg/kg) for 9 days, and on the 5th day, CIS was injected by i.p at a dose of 7 mg/kg. Group V: (CIS-NARNS) rats of this group were administered with NARNS (50 mg/kg). P; once daily) for 9 days and CIS at a dose of 7 mg/kg was injected by i.p on the fifth day of the treatment (Kapoor and Kakkar, 2014).

Animals were euthanized at the end of the experiment, under light anesthesia with diethyl ether; rats were euthanized by cervical decapitation. For biochemical assays, blood samples were collected, and serum was separated by centrifugation at 2500 rpm for 15 min. The samples were analyzed according to the prescribed procedure for required biochemical parameters. Total bilirubin and direct bilirubin was evaluated by Jendrassik & Grof’s method (Garber, 1981). Triglycerides activity of serum estimated using Glycero kinase peroxidase method. CHOD-PAP assay was preferred for the study of total cholesterol activity. Albumin and total protein were evaluated by the biuret method (Gornall et al., 1949). AST, ALT and ALP levels were assessed from the collected serum with commercially available kits by using an autoanalyzer (Garba et al., 2011). Livers were excised, rinsed, cleaned in saline and preserved in 10% formalin for histopathological study (Upreti et al., 1991).

Nephroprotective activity

Albino Wistar strain rats were divided into five groups of six each. Single-dose i.p. of CIS (7 mg/kg b.w.) was used to induce nephrotoxicity. Group I, the Control group animals, were selected as normal control. Group II-CIS treated group rats were administered with an intraperitoneal CIS i.p dose of 7 mg/kg on the fifth day from the start day of the experiment. Group III CIS-Cystone, rats of this group were administrated with Cystone (5ml/kg; p.o; once a day) for 9 days and on the 5th day CIS (7 mg/kg b w) was injected by i.p route (Rao and Rao, 1998; Sahoo et al., 2011). Group IV-CIS-NAR, rats of this group, were administered with NAR (50 mg/kg i.p; once daily) for 9 days and on the 5th day, CIS was injected by i.p at a dose of 7 mg/kg. Group V-CIS-NARNS rats of this group were administered with NARNS (50 mg/kg i.p; once daily) for 9 days, and CIS at a dose of 7 mg/kg was injected by i.p. On the fifth day of the treatment (Rajappa et al., 2017). Once the experiment was completed, rats were euthanized under light anesthesia with diethyl ether. Rats were euthanized by cervical decapitation. For biochemical assays, blood samples were collected and serum was separated by centrifugation at 2500 rpm for 15 min and investigated for various biochemical constraints such as Blood Urea Nitrogen by DAM method (Allston, 1993), Serum Creatinine by Jaffe’s Alkaline picate method (Vasiliades, 1976; Zaveri et al., 2011), and serum uric acid were estimated using commercial kits (Hooper et al.,
Tissue preparation

Dissected tissues were rinsed with cold PBS buffered solution (pH 7.4) and preserved at -80°C until the analysis. Tissue homogenate was prepared by homogenizing the tissue in a ratio of 1:3 (w/v; 1 g tissue with 3 ml PBS, pH 7.4) at 10000 rpm for 15 min at 4°C. The Clear supernatant was used for the determination of malondialdehyde (MDA) contents, reduced glutathione (GSH) and antioxidant enzyme activities.

Biochemical estimation of markers of oxidative stress in liver and kidneys

Estimation of lipid peroxidation levels

The process of lipid peroxidation is fixed in the supernatant collected after the homogenization of hepatic tissue by the thiobarbituric acid (TBA) method that figures the MDA formation. 1ml of 1% TBA and 50 μl of tissue homogenates were added with 500μl of 70% alcohol. The prepared samples in test tubes were subjected to boiling water for 20 min. 50 μl of acetone was added to all the samples once they were cooled and absorbance was determined at 535 nm in a spectrophotometer (Niehaus and Samuelsson, 1968).

Estimation of reduced glutathione levels

The activity of Glutathione peroxidase (GPx) was calculated as an alteration of the calorimetric method (Flohé et al., 1984), using hydrogen peroxide as a substrate in the existence of GSH. The absorbance was observed at 420 nm. Glutathione-s-transferase (GST) was determined by the ensuing the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm (Habig et al., 1974). Superoxide dismutase (SOD) activity was determined based on the capacity of SOD to inhibit the autoxidation of pyrogallol (Saggu et al., 1989). Catalase (CAT) activity was determined from the rate of decomposition of H2O2 by the described method (Bergmeyer and Gawehn, 1974).

Histopathological examination

Rats were sedated under light anesthesia with diethyl ether. The kidneys and liver of the animal were dissected out. The liver and kidney were further perfused with buffer saline to remove excess blood. Kidney was isolated and stored at -20°C. The kidney was stabilized with 10% neutralized buffered formalin. Fixed materials were implanted in paraffin wax for the sectioning of the sample in a thickness of 5μm thickness. Slides were stained with hematoxylin and eosin for histological study.

Statistical analysis

The value represents as mean ± SEM. The results were studied using a one-way ANOVA followed by Dunnet test. * p < 0.01, significant difference compared with the control group, * p < 0.05, significant difference compared with control group (n = 6).

RESULTS AND DISCUSSION

Parameters Assessed For Liver Functions

CIS administration resulted in significant elevation of bilirubin, triglycerides, total cholesterol, AST, ALT and ALP and bilirubin levels. Protein levels were significantly decreased in the test group compared to the normal control group indicating liver damage. Pretreatment with silymarin, NAR and NARNS significantly cause reduction of CIS-administered rats compared to the control group. Silymarin, NAR and NARNS significantly cause reduction of CIS-administered rats compared to the control group. NARNS at a dose of 50mg/kg notably (* p < 0.05; *p < 0.01) prevented hepatotoxicity induced by CIS. Data obtained are summarized into Table 1.

Estimation of lipid peroxidation levels

Degenerative properties of free radicals are well indicated with the lipid peroxidation of biomembranes. Levels of MDA in the above groups were observed. MDA level was markedly better in the CIS treated animals than those of the control group. Silymarin, NAR and NARNS significantly cause reduction of CIS-elevated MDA level compared to those of the CIS group.

Effects of NARNS on GSH contents

GSH is a sulphydryl-having compound which is responsible for holding homeosis of cellular oxidation-reduction. Alterations in GSH may be
Table 1: Effect of NAR and NARNS on liver function of CIS–induced liver injury in rats

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Total bilirubin (mg/dl)</th>
<th>Direct bilirubin (mg/dl)</th>
<th>Triglycerides (mg/dl)</th>
<th>Total cholesterol (mg/dl)</th>
<th>Total protein (g/dl)</th>
<th>Albumin (g/dl)</th>
<th>AST (U/L)</th>
<th>ALT (U/L)</th>
<th>ALP (U/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1.63± 0.02</td>
<td>0.50± 0.01</td>
<td>152± 3.24</td>
<td>206.5± 3.40</td>
<td>5.26± 0.06</td>
<td>103.83± 0.35</td>
<td>102.66± 1.88</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>3.82± 0.05</td>
<td>1.45± 0.03</td>
<td>238± 4.39</td>
<td>378.66± 4.75</td>
<td>2.75± 0.33</td>
<td>166.5± 273.15</td>
<td>215.0± 1.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2.2± 0.01</td>
<td>0.70± 0.01</td>
<td>166± 3.92</td>
<td>249.0± 7.23</td>
<td>4.87± 0.02</td>
<td>119± 102.2</td>
<td>161.0± 1.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>3.61± 0.03**</td>
<td>1.31± 0.02**</td>
<td>202± 4.96**</td>
<td>307.16± 6.33**</td>
<td>4.09± 0.07**</td>
<td>134.16± 190.1</td>
<td>194.66± 1.11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>3.14± 0.02**</td>
<td>0.99± 0.04**</td>
<td>182± 5.04**</td>
<td>279± 5.9±</td>
<td>3.75± 0.12**</td>
<td>128.5± 170.4</td>
<td>179.66± 6.12</td>
<td>1.54**</td>
<td></td>
</tr>
</tbody>
</table>

Value represents as mean ± S.E.M. **p<0.01, significant difference compared with the control group, *p<0.05, significant difference compared with control group (n=6).

Figure 1: A) Effect of NAR & NARNS on antioxidant activity. B) Effect of NAR & NARNS on liver function.
Table 2: Effect of NAR on liver tissue MDA, GSH levels and antioxidant enzyme activities of control and treated rats.

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>MDA (nmol /mg of protein)</th>
<th>GSH (U/mg of protein)</th>
<th>GPX (U/mg of protein)</th>
<th>GST (nmol /mg of protein)</th>
<th>SOD (U/mg of protein)</th>
<th>CAT (U/mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.376±0.006</td>
<td>0.33±0.01</td>
<td>24.45±0.20</td>
<td>0.033±0.001</td>
<td>21.25±0.24</td>
<td>55.45±0.23</td>
</tr>
<tr>
<td>II</td>
<td>0.558±0.006</td>
<td>0.10±0.007</td>
<td>10.65±0.09</td>
<td>0.015±0.0008</td>
<td>10.48±0.17</td>
<td>30.48±0.27</td>
</tr>
<tr>
<td>III</td>
<td>0.425±0.007</td>
<td>0.28±0.01</td>
<td>21.51±0.17</td>
<td>0.027±0.003</td>
<td>19.31±0.07</td>
<td>50.26±0.23</td>
</tr>
<tr>
<td>IV</td>
<td>0.496±0.006**</td>
<td>0.16±0.006**</td>
<td>16.51±0.09**</td>
<td>0.027±0.003</td>
<td>14.45±0.19**</td>
<td>37.56±0.24**</td>
</tr>
<tr>
<td>V</td>
<td>0.453±0.005*</td>
<td>0.22±0.007**</td>
<td>19.48±0.11**</td>
<td>0.024±0.0004*</td>
<td>17.75±0.18**</td>
<td>45.51±0.34**</td>
</tr>
</tbody>
</table>

Value represents as mean ± S.E.M. *p<0.05, significant difference compared with control group, **p<0.01, significant difference compared with 0.34 group (n=6).

reflected as a representation of functional cell damage. Levels of GSH in the above groups were noted. The toxicity of CIS appreciably reduced the levels of hepatic GSH in the CIS treated animals while comparison with the control group. Administration of silymarin, NAR and NARNS ameliorated the GSH levels in when compared with the CIS-treated group.

Effects of NARNS on antioxidant enzyme activities

The activities of GPx, GST, SOD and CAT of the studied liver tissue were shown in Table 2, Figure 2 A & Figure 2 B. Administration of CIS lead to a lower GPx, GST, SOD and CAT activities while comparison with the control animals. But animals treated with silymarin expressed obviously increase in antioxidant enzyme level followed by NARNS as compared to CIS group.

Histopathological examination of liver tissue

The examination of liver tissues of the control group exhibited normal liver cell with a nucleus, central veins and preserved cytoplasm (Figure 3 A). While the CIS treated rats where observed with liver sections where severe structural damage identified with necrosis around the central vein, degeneration and sinusoidal dilatation and inflammatory cell infiltration (Figure 3 B). Histological section of the liver of the rat treated with CIS and silymarin (10 mg/kg) showed (Figure 3 C) marked development in contrast with CIS control group, and it reverted the histological appearance seen in the latter group to normal in examined tissue fields. However, the histopathological studies of liver sections from NAR (50mg/ kg) treated animals, were observed with a moderate degree of liver damage. Lesser inflammation, reduced hepatocyte degradation and centrlobular necrosis were observed with NAR treated group Figure 3 D). The NARNS treated group was found with normal hepatocytes with least prominent inflammation.

(Figure 3 A) Normal sinusoidal space with normal morphology of hepatocytes with fine nuclear chromatin pattern. No perivenular inflammatory cell infiltration. (Figure 3 B) The histopathological observation in CIS treated rats liver shows sinusoidal space dilatation. Mild dilation and congestion of sinusoidal space seen with hemorrhage Degen-
eration of hepatic cells with glycogen depletion and coarse nuclear chromatin condensation. Focal perivenular inflammatory cell infiltration. (Figure 3 C) The treatment with silymarin group observed minimal sinusoidal space dilation. Mild dilation and congestion of sinusoidal space seen without hemorrhage. Regeneration of hepatic cells with glycogen depletion and fine nuclear chromatin. Minimal perivenular inflammatory cell infiltration. (Figure 3 D) NAR treatment on rat liver shows mild glomerular atrophy, less disarrangement of hepatocytes, minimal proximal tubular necrosis, as well as marked regeneration activity. (Figure 3 E) Almost normal sinusoidal space of the liver. Regeneration of hepatic cells seen with normal glycogen content and fine nuclear chromatin. Minimal perivenular inflammatory cell infiltration.

**Parameters Assessed For Kidney Functions**

Biochemical parameters such as blood urea nitrogen, Serum urea, uric acid and creatinine concentrations were significantly improved in the group treated with only CIS as matched with the normal control, on the other hand, groups treated with Cystone, NAR and NARNS showed significantly low values when equaled with the positive control group. NARNS was observed as predominant nephroprotective effect compared to NAR (*p < 0.05; * *p <
Figure 3: Photomicrographs of liver sections stained with hematoxylin–eosin below the light microscope

A: Normal  B: CIS  C: Silymarin

D: NAR  E: NARNS

Figure 4: Effect of NAR & NARNS on kidney function

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Histopathological examination of Kidney tissue

The sections of the kidney of normal control rats exhibited normal renal tubules and renal corpuscles. The glomeruli and Bowman’s capsule appeared to be normal and prominent (Figure 5 A). In the CIS control group, the kidney demonstrated discriminating tubular necrosis, dilated proximal convoluted tubules, plodding of epithelium due to desquamation and atrophic glomeruli, cellular debris in the tubular lumen and better tissue in the interstitium, were an indication of CIS-induced necrosis of kidney, which were signs of nephrotoxicity (Figure 5 B). A Histological section of the kidney of the rat treated with CIS and Cystone (5 ml/kg) showed an improvement while comparing the CIS control group, and it reverted the histological characteristics observed in the latter group to normal in examining tissue fields. There were areas of tubular injury with tubular atrophy, tubular necrosis, and interstitial fibrosis, but these factors had a lesser intensity than in CIS control group (Figure 5 C). Kidney histological sections of rats treated with CIS + NARNS (50 mg/kg) showed marked improvement, showing normal kidney histology and architecture, with interstitial fibrosis. There were some foci viewing tubular injury with, tubular atrophy, tubular necrosis and interstitial fibrosis, which were less intense than those seen in CIS control group and CIS + NAR (50 mg/kg) group (Figure 5 D).

(Figure 5 A) Normal glomeruli and tubules were observed in normal rat kidney. It shows a normal glomerulus, proximal and distal tubules. (Figure 5 B) The histopathological observation in CIS treated rats showed glomerular atrophy, significant proximal tubular necrosis, tubular edema, hydropic degeneration of tubular epithelial cells and edematous intertubular spaces. (Figure 5 C) The treatment with Cystone group observed normal glomeruli, edematous proximal tubules without necrosis, normal tubular epithelial cells. (Figure 5 D) NAR treatment on rat kidneys shows mild glomerular atrophy, minimal proximal tubular necrosis, tubular edema, normal tubular epithelial cells and edematous intratubular spaces. (Figure 5 E) Almost normal appearance of kidney glomeruli and tubules were regenerated in NARNS.

CONCLUSION

The study results clearly suggested that oxidative stress, inflammation and apoptosis/necrosis play a critical role in the pathogenesis of CIS induced hepato and nephrotoxicity. Pretreatment with NARNS significantly attenuated CIS-induced functional and histological liver and renal deterioration compared to NAR. One of the potential reason behind the
NARNS-mediated protection of the cells were maybe the antioxidant activity. Before CIS administration, pretreatment with NARNS could inhibit the origin of free radicals produced by CIS prior to accomplish DNA and causing damage. The study delivers a solid proof for the use of the NARNS as hepato and nephroprotective activity against CIS induced damage in the liver and kidney. Therefore, NARNS may act as a chemoprotective agent to protect the cells from CIS-mediated toxicity.

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