Resveratrol mitigate structural changes and hepatic stellate cell activation in N' nitrosodimethylamine-induced liver fibrosis via restraining oxidative damage

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Abstract
Resveratrol, a polyphenol, found in skin of red grapes, peanuts and berries possesses anti-inflammatory, anti-carcinogenic and lipid modulation properties. Here, we demonstrate in vivo antifibrotic activity of resveratrol in a mammalian model, wherein hepatic fibrosis was induced by N'-nitrosodimethylamine (NDMA) administration. Apart from being a potent hepatotoxin, NDMA is a known mutagen and carcinogen, as well. To induce hepatic fibrosis, rats were administered NDMA (i.p.) in 10 mg/kg b.wt thrice/week for 21 days. Another group of animals received resveratrol supplement (10 mg/kg b.wt) subsequent to NDMA administration and were sacrificed weekly. The changes in selected biomarkers were monitored to compare profibrotic effects of NDMA and antifibrotic activity of resveratrol. The selected biomarkers were: sera transaminases, ALP, bilirubin, liver glycogen, LPO, SOD, protein carbonyl content, ATPases (Ca\(^{2+}\), Mg\(^{2+}\), Na\(^{+}/K\(^{+}\)) and hydroxyproline/collagen content. Alterations in liver architecture were assessed by H&E, Masson's trichrome and reticulin staining of liver biopsies. Immuno-histochemistry and immunoblotting were employed to examine expression of \(\alpha\)-SMA. Our results demonstrate that during NDMA-induced liver fibrosis transaminases, ALP, bilirubin, hydroxyproline/collagen and liver decreases, while liver glycogen is depleted. The decline in SOD (>65%) and ATPases, which were concomitant with the elevation in MDA and protein carbonyls, strongly indicate oxidative damage. Fibrotic transformation of liver in NDMA-treated rats was verified by histopathology, immuno-histochemistry and immunoblotting data, with the higher expressivity of \(\alpha\)-SMA-positive HSCs being most established diagnostic immuno-histochemical marker of HSCs. Resveratrol-supplement refurbished liver architecture by significantly restoring levels of biomarkers of oxidative damage (MDA, SOD, protein carbonyls and membrane-bound ATPases). Therefore, we conclude that antifibrotic effect of resveratrol is due to restrained oxidative damage and down-regulation of \(\alpha\)-SMA, which inhibits HSC activation to obstruct liver fibrosis.

1. Introduction

Resveratrol (trans-3, 4, 5′-trihydroxystilbene) is a naturally occurring polyphenol and a potent phytoalexin. It is found in many spermatophytes such as peanuts, skin of red grapes, berries and roots of Japanese knotwood, Polygonum cuspidatum [1]. Modulatory actions of resveratrol in cell involve interactions with a number of enzymes central to the cell signaling pathways. These include lipoxygenase, cyclooxygenase (COX-1, COX-2), protein kinases, nitric oxide synthase, ribonucleotide reductase and P450 [2–7]. Of specific interest to this study are anti-inflammatory and anti-oxidative properties of resveratrol (Rsvtr), through which it protect cells from reactive oxygen species (ROS-inflicted oxidative damage). Some of the interesting studies suggest that the presence of three hydroxyl groups (′OH) in trans-resveratrol counters several types of oxidative damages [8–11], and also confers radical scavenging activity that protects phospholipids of hepatic bio-membranes from peroxidative damage.

Reports that in cell culture, oxidative stress-mediated abnormal proliferation of hepatic stellate cells (HSCs) results in increased collagen synthesis have prompted further investigations on the effects of oxidative damage in activation process of stellate cells [12,13]. Moreover, published evidence strongly suggests a correlation between liver fibrosis and oxidative stress [14,15]. Therefore, oxidative stress does have a bearing on the activation of HSCs [16]. Oxidative stress leading to production of reactive oxygen species (ROS) is known to play a crucial role in many types of liver
injuries [14,17]. *N*-Nitrosodimethylamine (NDMA), a potent hepatocarcinogen and mutagen, induces not only liver fibrosis but also cirrhosis consequent to repeated exposure of animals to its lower dosages [18–20]. NDMA stimulates the generation of free radicals and ROS by hepatocyte, Kupffer cells and neutrophils adding further to oxidative damage [21,22]. The net consequence of oxidative damage would be the impairment in phospholipids biomembranes and vital macromolecules like proteins and DNA, which causes gross alterations in cellular morphology and functioning with varying degrees of necrosis, fibrosis and nodular regeneration in liver. Due to the crucial role of ROS in liver pathology, antioxidants are likely to avert liver damage by increasing the intracellular antioxidant milieu.

Various naturally occurring compounds of plant origin have shown strong antioxidant activity which can possibly be tagged as prospective pharmaceuticals with potential application as hepatoprotective and antifibrotic agents in combating liver ailments [19,23–27]. Since inflammatory mediators stimulate HSC activation [28], it is conceivable that it is primarily via inhibition of HSC activation pathway that resveratrol exerts its antifibrotic effect in mitigating liver injury. Only a couple of reports have so far shown antifibrotic activity of Rsvtrl by relieving rat liver injury, induced selectively by chronic biliary obstruction or CCl₄ [9,29] or in hypercysteinemic mice [30]. The new evidence on antioxidant potential of Rsvtrl obtained in our study differs in design and scope from the above cited work on liver injury and in this way it appears to be the first report to show inhibitory role of Rsvtrl on HSC activation in vivo. We emphatically demonstrate that Rsvtrl mitigates HSC activation in vivo and restores liver architecture by alleviating rat liver from NDMA-induced oxidative damage.

2. Materials and methods

2.1. Chemicals

*N*-Nitrosodimethylamine (NDMA), Adenosine 5′-triphosphate (ATP) disodium salt hydrate, resveratrol, 2-Thiobarbituric acid (TBA), 2,4-dinitrophenylhydrazine (DNPH) and guanidine hydrochloride were purchased from Sigma–Aldrich. Pyrogallol, Dextrose (TBA), 2,4-dinitrophenylhydrazine (DNPH) and guanidine hydrochloride were prepared using kits from Erba Diagnostics Mannheim Gmbh (Mumbai, India), Coral Clinical Systems (Goa, India) and AutoZyme (Accurex Biomedical Pvt. Ltd, Mumbai, India). The biomarkers were: sera alkaline phosphatase (SALP), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and bilirubin.

2.2. Animals

Six to eight week old, adult male albino rats (*Rattus norvegicus*) of Wistar strain weighing around 160 ± 10 g, procured from the Institutional Animal House Facility, were used in the present study. The animals were maintained at 12 h light: dark cycle in sterilized polycarbonate cages with wire mesh top and a bed of husk under proper hygienic conditions in animal house facility. The rats were acclimatized for a week before the start of the treatment while being regularly fed with commercial, sterilized diet (Ashirwad Industries Private Ltd., Mohali, Punjab, India) and water ad libitum. Animal care and experiments were performed according to the guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPSEA), India.

2.3. Study design

Animals were divided into four groups comprising of fifteen rats each (*n* = 15). Group I served as saline control and received normal saline (10 mL kg⁻¹ i.p.); Group II rats (Rsvtrl group) were given only resveratrol, in doses of 10 mg kg⁻¹ body weight (i.p.); Group III was treated with NDMA (10 μL diluted to 1 mL with 0.15 M sterile NaCl) in doses of 10 mg kg⁻¹ body weight (i.p.) and Group IV animals, were given resveratrol (10 mg kg⁻¹ body weight) after 2 h lag of administering NDMA in above mentioned dosage. The 2 h lag of resveratrol administration was selected on the basis of pilot experiments. The dose of resveratrol was narrowed down on the basis of previously published reports [29,31,32] and some initial experiments. The injections were given on three consecutive days of each week for three weeks, as described earlier [19,33]. Five animals from each group were anaesthetized and sacrificed weekly on day 7, 14 and 21 from the start of treatment. Animal body and liver weight were monitored throughout the experiment.

2.4. Sample collection and preparation

Before sacrifice, blood was collected through cardiac puncture on weekly basis i.e. on days 7, 14 and 21 from both treated as well as control groups, from the beginning of experiment. Blood samples were kept at room temperature for an hour to ooze out sera. Samples were centrifuged at 3000 rpm and 4 °C for 8–10 min and the resulting clear, pale colored sera was either analyzed afresh or stored in aliquots at −20 °C for further biochemical investigation. Liver from sacrificed rats of each category were quickly excised with sterilized scissors and forceps, washed with phosphate buffered saline (PBS) to remove other tissue debris. Respective liver samples, unless otherwise specified, were homogenized in 1.3 (w/v) Tris–HCl buffer (50 mM, pH 7.5). The crude homogenates were centrifuged at 8000 rpm and 4 °C for 30 min and the clear supernatant were saved for biochemical assays. In addition to homogenate, a piece of liver tissue (from each animal) was fixed in formalin (10%) and processed for histopathological studies as described under appropriate subheading, below.

2.5. Assay of liver function biomarkers

The activities of biomarker of liver function (LFT) were determined using kits from Erba Diagnostics Mannheim Gmbh (Mumbai, India), Coral Clinical Systems (Goa, India) and AutoZyme (Accurex Biomedical Pvt. Ltd, Mumbai, India). The biomarkers were: sera alkaline phosphatase (SALP), Aspartate aminotransferase (AST), Alanine aminotransferase (ALT) and bilirubin.

2.6. Total protein estimation

Protein concentration of sera and liver homogenates were determined employing the method of Lowry et al. [34] using Folin and Ciocateu’s Phenol as color reagent and known concentration of bovine serum albumin as standard. Optical density (absorbance) was recorded at 660 nm on a GeneSys 10 UV-Scanning spectrophotometer.

2.7. Glycogen estimation

For glycogen estimation, 200 mg of liver tissue was homogenized in 20 mL of 5% trichloroacetic acid. The homogenate thus prepared, was centrifuged at 5000 rpm and 4 °C for 20 min and the clear supernatant (liver extract) was saved. Liver extract (~2 mL) was mixed with equal volumes of 10 N KOH and kept in boiling water bath for 1 h. The content was brought down to RT and excess alkali was neutralized by adding 1 mL of acetic acid. The volume was finally raised to 20 mL with distilled water. For color development, the above solution was mixed with freshly prepared anthrone reagent (0.2% w/v in 95% H₂SO₄) in 1:2 proportions under ice. After thorough mixing, the samples were incubated in
boiling water bath for 10–15 min [35]. The samples were read at 650 nm against blank lacking glycogen source using glucose standards.

2.8. Determination of lipid peroxidation

Lipid peroxidation was determined by thiobarbituric acid (TBA) reaction in terms of malonaldehyde (MDA) following the method of Ohkawa et al. [36]. Briefly, the reaction mixture contained 0.2 mL of crude liver homogenate, 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid (pH 3.5) and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid (TBA). The mixture was finally made up to 4 mL with distilled water and heated at 95 °C for 1 h. After cooling, 5 mL of n-butanol and pyridine mixture (15:1 v/v) was added to it and vortexed. The solution was centrifuged at 8000 rpm for 10 min. The absorbance of organic layer was recorded at 532 nm, MDA, a diagnostic measure of lipid peroxidation, was calculated using an extinction coefficient of 1.56 × 10^5 M^{-1} cm^{-1}.

2.9. Estimation of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity in the liver homogenates was estimated employing the protocol of Marklund and Marklund [37]. Briefly, the reaction mixture consisted of liver sample (50 μL), 0.2 M Pyrogallop, 1 mM EDTA and 50 mM Tris–HCl (pH 8.2) in a final volume of 1 mL. The enzymatic activity was measured by monitoring the changes in absorbance for 3 min at 420 nm. 50% inhibition in Pyrogallop auto-oxidation is considered a unit of enzyme activity [38].

2.10. Determination of total carbonyl content

Protein carbonyls were determined employing the method of Levine et al. [39] using 2,4-dinitrophenylhydrazine (DNPH). Briefly, 2 mL DNPH (in 2.5 M HCl) was mixed with 500 μL of tissue homogenate and incubated at the room temperature for 1 h. Then, protein was precipitated by adding 2 mL of 20% trichloroacetic acid and washed thrice with 2 mL of ethanol: ethyl acetate (1:1). Following centrifugation, collected precipitate was redissolved in 1 mL of 6 M guanidine HCl in 20 mM potassium phosphate (pH, 6.5). Total carbonyl content was calculated from the absorbance recorded at 380 nm against a blank using a molar absorption coefficient of 22,000 M^{-1} cm^{-1} and expressed as nmol carbonyl mg^{-1} protein.

2.11. Assay of membrane-bound ATPases

Activity assays for membrane ATPases in liver samples were carried out at 20 °C [40] and liberated inorganic phosphate was calculated by the method of Fiske and Subbarow [41]. Ca^{2+}-ATPase activity was assayed in a reaction mixture (2 mL) that at final concentration contained 5 mM CaCl₂, 1 mM ATP, 20 mM Tris–HCl buffer (pH, 7.5) and 0.2 mL of liver homogenate. Mg^{2+}-ATPase activity was estimated as described above, except that MgCl₂ of the same molarity instead of CaCl₂ was used in the reaction mixture. For Na⁺/K⁺-ATPase activity determination, in addition to tissue homogenates (0.2 mL) and ATP (2 mM), 0.32 mL Tris–HCl buffer (250 mM, pH, 7.5), 0.1 mL MgCl₂ (0.1 M), 0.32 mL NaCl (0.5 M) and 0.4 mL KCl (0.1 M) was added. Following incubation at 20 °C for 20 min, absorbance was recorded at 640 nm on spectrophotometer and the ATPases activity was calculated using sodium dihydrogen phosphate standard.

2.12. Hydroxyproline and collagen measurement

Levels of hydroxyproline (HP) were measured in sera and liver of control and treated group of rats essentially by the protocol of Woessner [42]. The collagen content in the liver was calculated by multiplying the obtained hydroxyproline value (in liver) by a factor of 7.46, as described by Neuman and Logan [43].

2.13. Histopathology

The standardized protocol of Ahmad et al. [19] was followed for histopathology. Briefly, the formalin-fixed liver tissues were dehydrated and embedded in paraffin as blocks and processed for microtomy. The progression of liver fibrosis and the effect of Rsvtrl supplement was monitored by hematoxylin and eosin (H&E), Mason’s trichrome and reticulin staining of 5 μm thick serial liver sections. Stained slides were examined and photographed under Nikon microscope with an LCD attachment (Model: 80i).

2.14. α-Smooth muscle actin (α-SMA) localization by immunohistochemistry

Serial liver sections were incubated with 3% H₂O₂ for 15 min to quench endogenous peroxidase activity. Sections were then washed with PBS before layering with pre-diluted monoclonal α-SMA antibodies and kept overnight in a moist chamber at 4 °C. Unbound antibodies were washed off with PBS and the slides were incubated with HRP-conjugated goat anti-mouse IgG (secondary antibody) for 40–50 min at room temperature. Following washing with PBS, liver sections were stained with 3,3’-Diaminobenzidine tetrachloride hydrate (3% w/v) for 10–15 min, rinsed with PBS, counter-stained with Mayer’s hematoxylin and mounted with DPX for further investigations. Selected slides from control and treated groups were photographed under microscope at desirable magnifications.

2.15. Polyacrylamide gel electrophoresis (PAGE) and western immunoblotting

Polyacrylamide gel electrophoresis (PAGE) was performed according to the protocol of Laemmli [44] with slight modifications. Briefly, the gels contained acrylamide (acrylamide: bis-acrylamide = 29.2:0.8), 0.1% sodium dodecyl sulfate (SDS) and 10% glycerol. SDS was also added to the gels, samples and running buffers in its prescribed concentration. Dimensions of the slab gels were 100 × 150 × 1 mm. Lower gels (10%) were made in 0.375 M Tris–HCl (pH, 8.8) and upper gel (3%) in 0.125 M Tris–HCl (pH, 6.8). Runs were made in Tris–glycine (24 mM and 194 mM, respectively) of pH 8.3 containing 0.1% SDS (w/v). The runs were made at 60 V, 15 mA/gel for 3 h at RT. The gels were washed in 7% acetic acid prepared in 10% methanol to remove SDS and stained with CBBR250. Electrophoretic patterns were documented under visible range illumination using SONY Cybershot digital camera (14.1 Megapixel, 4X optical zoom). Molecular weight (Mr) of various molecular weight markers was estimated by GelPro software (Media cybernetics, USA) analyses. Freshly prepared chicken actomyosin was used as a molecular weight marker.

Western immunoblotting was performed according to the methodology of Sambrook et al. [45] with minor modifications as reported earlier [46]. Separate runs of SDS–polyacrylamide (SDS–PA) gels were equilibrated in Tris–glycine transfer buffer (24 mM Tris, 194 mM Glycine and 10% v/v Methanol) for 25–30 min. The electrotransfer was carried out on PVDF membranes (0.45 mm, BioRad, USA) at 125 V/200 mA for 2 h at 4 °C. Following membranes, were washed thrice in PBS (50 mM, pH 7.1). The membrane was treated with biotin (5% w/v non-fat dry milk in PBS) for 1 h at RT. All further incubations and washings were carried out in the same blocking solution also containing 0.1% Tween-20 (PBS-T) (w/v). The blot was then incubated for 2 h in primary antibody (α-smooth muscle actin monoclonal antibodies) diluted to 1:250, with
constant shaking. After three washes with PBS-T, the membrane was treated with horseradish peroxidase-conjugated goat anti-mouse IgG (secondary antibody) diluted to $\times 200$, for 1 h. Subsequently, the membrane was washed thrice with PBS-T to remove the unbound antibody. Chemiluminescence detection of peroxidase activity was performed with LumiGlo Chemiluminescent kit (KPA, USA) wherein the membrane was incubated with luminal reagents, exposed to sensitive USG films for ~5 min under dark and developed. Densitometric analysis of the selected blot-scans was carried out using Scion Imaging (Scion Corporation, Beta release, 4.0) and GelPro (Media cybernetics, USA) software programs.

2.16. Statistical analysis

Values for all the biochemical data are expressed as mean value ± SD. Statistical analysis was performed by one-way analysis of variance (ANOVA) using SPSS 16.0 software (SPSS Inc., USA). The statistical significance between the experimental groups was assessed by LSD ALPHA (0.05) post hoc test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Change in animal body weight and hepato-somatic index (HSI)

The differences in the mean body weight and HSI of control (saline), Resveratrol (Rsvtrl)-alone, NDMA- and NDMA + Rsvtrl-supplemented groups of animals are given in Table 1. NDMA intoxication significantly decreased animal body and liver weight, which was primarily prevented by the administration of Rsvtrl supplement ($P < 0.05$).

3.2. Indicators of liver function

In the NDMA-intoxicated group, activities of serum ALP, AST and ALT were significantly higher ($P < 0.05$) than the control group, indicating NDMA-mediated disruption in protein synthesis in the liver ($P < 0.05$). On the contrary, Rsvtrl-supplement markedly suppressed these NDMA-induced elevations in the levels of serum ALP, ALT and AST. Moreover, Rsvtrl restrained the NDMA-mediated increase in serum bilirubin (DBil/TBil) contents (Table 1).

3.3. Glycogen, LPO, SOD, carbonyl contents and membrane bound ATPase activities

Liver glycogen, the major source of energy in an organism, was markedly reduced during the course of NDMA-treatment in rats. On the contrary, Rsvtrl-supplement significantly countered this NDMA-mediated depletion of liver glycogen indicating enhanced glycogenesis or reabsorption by the hepatocytes ($P < 0.05$). In comparison, the Rsvtrl group showed no alterations in the levels of liver glycogen (Fig. 1A).

The extent of lipid peroxidation (LPO) was measured by determining malonaldehyde (MDA) levels in liver homogenates. Oxidative damage induced by NDMA is evident by the incessant increase in levels of MDA formation in liver during the course of treatment, which eventually reached to almost threefold increase by the end of NDMA-treatment (Fig. 1B). Oxidative damage in rats was further corroborated by more than 65% decline in liver SOD ($P < 0.01$) activity (Fig. 1C), approximately threefold increase in total carbonyl content (Fig. 1D) and a decrease up to 32%, 26% and 23% in Ca$^{2+}$-, Mg$^{2+}$- and Na$^+$K$^+$ ATPases activities respectively ($P < 0.05$) in liver tissues after 21 days of NDMA treatment as compared to control (Fig. 2A–C). Rsvtrl-supplement in the prescribed dosage significantly curtails the formation of MDA, restores the levels of SOD and protein carbonyls thus exhibiting antioxidant potential against NDMA-induced oxidative damage. No significant difference was observed in these parameters between the control groups.

3.4. Estimation of hydroxyproline and collagen contents

Sera content of hydroxyproline, an amino acid exclusively present in collagen, showed significant differences among different treatment groups. In liver, the levels of hydroxyproline were found to be significantly elevated during NDMA-intoxication, reaching more than 2.5-folds by the end of NDMA treatment ($P < 0.05$). The elevation, thus observed, demonstrated extensive collagen accumulation in the liver. On the contrary, the Rsvtrl treatment considerably minimized the NDMA-induced elevated hydroxyproline contents of sera/liver, suggesting a noticeable decline in collagen deposition (Table 2; Fig. 3).

3.5. Liver histology and immunohistochemistry

H&E stained liver sections showed normal liver architecture with central veins and radiating hepatic cords in control group of animals (Fig. 4A and E). Diffuse centrilobular congestion with marked dilation of central veins and hemorrhage was noticed in day 7 specimen of NDMA-treated liver (Fig. 4B). Centrilobular necrosis with marked neutrophilic and mononuclear cell infiltration along with mild bile duct hyperplasia (Fig. 4C) was seen in day 14 liver of NDMA-treatment. Intense fibrosis, with severe disruption of lobular architecture and abnormal collagen deposition was distinct on day-21 of NDMA treatment (Fig. 4D). Histological disruptions were remarkably reduced in the liver sections of resveratrol-supplemented rats. Rsvtrl treatment suppressed the levels of

Table 1

<table>
<thead>
<tr>
<th>Treatment category</th>
<th>Control (saline)</th>
<th>Rsvtrl (control)</th>
<th>NDMA (day 21)</th>
<th>NDMA + Rsvtrl (day 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>$162 \pm 10$</td>
<td>$160 \pm 08$</td>
<td>$133 \pm 10$</td>
<td>$151 \pm 08$</td>
</tr>
<tr>
<td>Hepato-somatic index (g%)</td>
<td>$4.854 \pm 0.32$</td>
<td>$4.81 \pm 0.28$</td>
<td>$3.525 \pm 0.286$</td>
<td>$4.56 \pm 0.38$</td>
</tr>
<tr>
<td>Serum ALP (UI/L)</td>
<td>$337 \pm 11.66$</td>
<td>$341 \pm 12.53$</td>
<td>$719 \pm 16.44$</td>
<td>$384 \pm 14.86$</td>
</tr>
<tr>
<td>AST/GOT (UI/L)</td>
<td>$33 \pm 7.96$</td>
<td>$33 \pm 8.52$</td>
<td>$188 \pm 5.86$</td>
<td>$143 \pm 8.86$</td>
</tr>
<tr>
<td>ALT/GPT (UI/L)</td>
<td>$59 \pm 6.32$</td>
<td>$61 \pm 7.22$</td>
<td>$73 \pm 7.36$</td>
<td>$65 \pm 6.64$</td>
</tr>
<tr>
<td>TBil (mg/dL)</td>
<td>$0.321 \pm 0.06$</td>
<td>$0.326 \pm 0.05$</td>
<td>$1.372 \pm 0.04$</td>
<td>$0.475 \pm 0.06$</td>
</tr>
<tr>
<td>DBil (mg/dL)</td>
<td>$0.142 \pm 0.021$</td>
<td>$0.144 \pm 0.011$</td>
<td>$0.157 \pm 0.03$</td>
<td>$0.149 \pm 0.02$</td>
</tr>
<tr>
<td>TSP (g/dL)</td>
<td>$7.76 \pm 0.62$</td>
<td>$7.72 \pm 0.77$</td>
<td>$6.92 \pm 0.8$</td>
<td>$7.48 \pm 0.44$</td>
</tr>
</tbody>
</table>

ALP = alkaline phosphatase; AST/GOT = aspartate aminotransferases/glutamic oxaloacetic transaminase; ALT/GPT = alanine aminotransferases/glutamic pyruvic transaminase; TBil = total bilirubin; DBil = direct bilirubin; TSP = total sera proteins.

* $P < 0.05$

** $P < 0.005$

† Significantly different from NDMA-treated group, $P < 0.05$. 

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< insertion of references and other details >
liver damage in a time and dose dependent manner, which is evident in rat liver sections of day 14 and 21 by minimal inflammatory cells infiltration; mild necrosis and regenerating hepatocytes (Fig. 4G and H).

The extent of liver damage (fibrosis) and the effect of Rsvtrl supplement was further documented using Masson’s trichrome (Fig. 5A–H) and reticulin (collagen type-III) staining (Fig. 6A–H) of liver biopsies. Bridging fibrosis and necrosis was noted during progressive stages of NDMA-treated liver specimens of rats (Fig. 5A–H). Day 21 liver sections demonstrated fibrosis with the deposition of collagen fibers surrounding the lobules, forming large fibrous septa (Figs. 5D and 6D). Thickenig and deposition of these collagen fibers was markedly reduced in Rsvtrl-supplemented rats, depicting the potential antifibrotic role of resveratrol.

The localization of α-smooth muscle actin (α-SMA), an indicator of activated HSCs, was carried out by the immunohistochemical staining of liver biopsies. In contrast to the control livers where α-SMA-positive cells were scarcely present around portal veins and hepatic arteries (Fig. 7A and E), many α-SMA-positive cells were seen around the peribiliary and parenchymal areas and were scattered in the regions of connective tissue septa of NDMA-treated rats (Fig. 7B–D). Rsvtrl administration along with NDMA suppressed the activation of HSCs and consequently, only traces of α-SMA positive stained cells were observed.

3.6. Polyacrylamide gel electrophoresis and western immunoblotting

Fig. 8A demonstrate typical PAGE patterns of low ionic strength soluble fractions of rat liver under denatured conditions. In SDS–PAGE profile, owing to their identical molecular weight, α-SMA and β-actin co-stack as a single band of ~44 kDa. Fig. 8B shows immunoblots of α-SMA in various treatment categories of animals. Expression of α-SMA in liver homogenates of rats treated with NDMA was significantly elevated as compared to the controls (P < 0.05). However, Rsvtrl-supplement effectively reduced the expression of α-SMA in rat liver (Fig. 8B). Densitometry of the blot-scans also confirmed that NDMA-mediated increase in α-SMA expression was reduced to nearly 50% by Rsvtrl treatment within 21 days.

4. Discussion

N’-Nitrosodimethylamine (NDMA) induced liver fibrosis in rats is one of the most extensively studied experimental model for elucidating the mechanism of liver fibrosis (LF). The model is equally suitable for evaluating the efficacy and hepatoprotective potential of a variety of natural and synthetic compounds, since it imitates fibrosis in human liver [19,47–49]. The biomarker estimates made in this study, combined with liver histology (using H&E and Masson’s trichrome, reticulin and immunological staining) and Western blotting, specifically demonstrate oxidative damage that occurred due to NDMA-intoxication and activation of HSCs. That resveratrol (Rsvtrl) was able to revert above mentioned damages to near normal biomarker levels or histological integrity, supports its therapeutic potential. To substantiate results specifically on oxidative damage, superoxide dismutase (SOD) activity, lipid peroxidation (LPO) and protein carbonyl contents were determined. The membrane bound ATPase activities (Ca$^{2+}$/Mg$^{2+}$/Na$^+$/K$^+$) were also assayed and for the first time presented in this study as the corroborative evidence.
As for some basic parameters, NDMA-induced reduction in the body weight and hepato-somatic index, its correlation with the cellular necrosis, altered metabolism, nutrient absorption and utilization data of this study, is in agreement with previous reports [31,50–52]. It is widely accepted that liver function enzymes seep out into sera when the affected tissue has suffered severe damage, which in the present case is fibrotic liver. Furthermore, we noticed that the decline in glycogen levels is concomitant with the rise in levels of liver function enzymes during NDMA-induced progressive LF. Nonetheless, the novel aspect of the present findings is that Rsvtrl mitigates LF, which is evident by significant reversal of loss in body weight, HSI and restoration of sera ALP, ALT and AST activities, when compared with NDMA-treated fibrotic rats. Here, we can underline two mechanisms which apparently restore glycogen reserve as the result of protective action of Rsvtrl: (1), it re-establishes normal absorption of glycogen by hepatocytes in fibrotic rats; or (2), by regaining storage ability of glycogen that necrotic cells had lost during NDMA-induced LF [49,53]. Owing to ready absorption of Rsvtrl in doses below 300 mg kg⁻¹ day⁻¹ (i.e. no observed adverse effect level or NOAEL) non-significant differences were observed in control groups. Available reports suggest that absorbed Rsvtrl is transported to various organs and metabolized to glucuronide and sulfate conjugates by different rodent species [32,54]. Since no report suggests diminished absorption of Rsvtrl in the doses administered here, it appears that absorption of Rsvtrl in rats is perturbed due to NDMA intoxication.

Table 2
Effect of resveratrol treatment on hydroxyproline levels in sera and liver tissues of rats treated with NDMA. Values are expressed as mean ± S.D (n = 5) of experiments performed in duplicates.

<table>
<thead>
<tr>
<th>Treatment Category</th>
<th>NDMA</th>
<th>NDMA + Rsvtrl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sera (µg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.22 ± 0.94</td>
<td>10.32 ± 0.65</td>
</tr>
<tr>
<td>Day 7</td>
<td>14.98 ± 0.63</td>
<td>13.226 ± 0.43</td>
</tr>
<tr>
<td>Day 14</td>
<td>12.68 ± 0.584</td>
<td>11.03 ± 0.509</td>
</tr>
<tr>
<td>Day 21</td>
<td>15.46 ± 0.522</td>
<td>10.86 ± 0.487</td>
</tr>
<tr>
<td>Liver (mg/g tissue)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.194 ± 0.0075</td>
<td>0.196 ± 0.0111</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.286 ± 0.022</td>
<td>0.212 ± 0.0144</td>
</tr>
<tr>
<td>Day 14</td>
<td>0.406 ± 0.006</td>
<td>0.298 ± 0.0147</td>
</tr>
<tr>
<td>Day 21</td>
<td>0.576 ± 0.0115</td>
<td>0.346 ± 0.0118</td>
</tr>
</tbody>
</table>

* P < 0.05.
** P < 0.01 vs. control (saline).
† Significantly different from NDMA-treated group, P < 0.05.
Oxidative stress, either mediated by reactive oxygen species (ROS) or free radical species, results in the oxidative decomposition of polyunsaturated fatty acids of membrane phospholipids that lead to the formation of a complex mixture of aldehydic end products such as malondialdehyde (MDA), 4-hydroxy-2,3-nonenal (HNE) and other 4-hydroxy-2,3-alkenals. ROS and free radicals, generated during cytochrome P450-mediated NDMA metabolism, have strong potential to react with triglycerides (lipids) of the hepatic biomembranes and would inflict peroxidative damage to produce lipid peroxides. Our data on liver of NDMA-treated rats

Fig. 4. Hematoxylin and Eosin (H&E) staining. H&E staining of serial liver sections during NDMA-induced hepatic fibrosis and subsequent treatment with resveratrol. (A) Control liver showing central vein and typical lobular architecture (10×). (B) Day-7. Peripheral lymphocyte infiltration (10×). (C) Day-14. Demonstrating Kupffer cell hyperplasia and fibrosis around the central vein (10×). (D) Day-21. Disrupted liver architecture, lymphocyte infiltration and well developed fibrosis (10×). (E) Control liver with normal structure subsequent to Rsvtrl treatment only (40×). (F) Day-7. Liver showing lymphocyte and neutrophilic infiltration with mild fibrosis (20×). (G) Day-14. Inflammation absent with decreased fibrosis along the central vein (10×). (H) Day-21. Restoration of normal liver architecture with increasing density of regenerating hepatocytes (10×).
showed significant increase in MDA (a secondary product of lipid peroxidation-mediated oxidative stress) levels (Fig. 1B) along with a drop in the activities of Ca$^{2+}$-, Mg$^{2+}$-, and Na$^+$/K$^+$-ATPases ($P < 0.05$) (Fig. 2A–C). Both of these parameters are indicators of oxidative stress and free radical inflicted disruption of hepatocellular membrane integrity, which is penultimate to hepatocytes damage. Moreover, HNE and MDA, being potential profibrotic mediators, have been correlated with active fibrogenesis by stimulating procollagen type-I synthesis and initiating the activation of HSCs [55–59]. The data obtained here on liver and sera hydroxyproline contents support the active hepatic collagenolysis during NDMA treatment [19,49,60–63]. Besides, significantly decreased SOD levels and elevated protein carbonyls ($P < 0.05$) furnish strong evidence for impairment in oxidative balance due to NDMA-intox-
The present results on SOD activity and protein carbonyls (as a result of oxidative damage to the protein) during NDMA induced LF are in concordance with the published reports [31,64,65]. Rsvtrl-supplement significantly regulated the NDMA-mediated alterations in above mentioned biomarkers of hepatic fibrosis in dose-dependent manner within three weeks. It is thus evident from our data that antioxidant activity of Rsvtrl substantially contributes to hepatoprotection that is rendered by this polyphenol. Hence, with the established association between oxidative stress and HSC activation in view [16,66], the evidences presented here underlines therapeutic efficacy of Rsvtrl in suppressing oxidative-stress during HSC activation pathway (Fig. 1B–D). Since the expression of antioxidant and detoxification enzymes such as SOD is under regulation of Nrf2 transcription factor [67], therefore, it appears to be a likely route of Rsvtrl-mediated detoxification of hepatocytes, which warrants further investigations.

Fig. 6. Reticulin staining of representative slides of liver sections. (A) Liver from saline control rat (10×). (B) Day-7. Reticulin deposition around the central veins (10×). (C) Day-14. Typical fibrotic appearance, thick fibrous septa showing the formation of bridging fibrosis between from central regions and portal areas (10×). (D) Day-21. Disrupted liver architecture with prominent intralobular inflammatory reaction consisting of granulocytes and mononuclear inflammatory cells in a well developed fibrotic nodule (40×). (E) Rsvtrl-control liver with normal lobular structure (10×). (F) Day-7. Liver showing minimal accumulation of collagen fibers (10×). (G) Day-14. Reduced inflammation with decreased collagen deposition (10×). (H) Day-21. Restoration of normal liver architecture (10×).
The above discussed results are unambiguously verified by histopathological data presented here, which clearly demonstrates disruption in liver architecture and deposition of collagen in the sinusoidal spaces of the liver in NDMA-treated rats (Figs. 4–6). Immuno-histochemistry and immunoblots show enhanced expression of α-SMA, specifying HSC activation in NDMA-induced fibrotic rats. During liver fibrosis, HSCs activation results in transdifferentiation to proliferating myofibroblasts by undergoing phenotypic modifications. This ultimately led to an abnormal ECM protein synthesis, in particular, collagen type II as well as
inhibiting its degradation together with de novo expression of α-SMA gene. In the liver of rats given Rsvtrl-supplement, α-SMA level declines significantly (Figs. 7 and 8). These inhibitory events reaffirm the potential antioxidant, anti-inflammatory and hepatoprotective role of Rsvtrl which is mediated via regulating the oxidative damage and α-SMA gene expression.

In conclusion, the antifibrotic effect of Rsvtrl is associated with its ability to repress oxidative damage by restoring levels of SOD, MDA (LPO), membrane-bound ATPases, the decline in protein carbonyls and inhibiting HSC activation by down regulating α-SMA expression. The present findings thus bring to focus antioxidant and anti-inflammatory properties of Rsvtrl and recognize its therapeutic potential as a bioagent for use in pharmaceutical preparations to combat liver fibrosis.

Fig. 8. α-Smooth muscle actin expressions in liver. (A) Typical SDS–PAGE profile of liver homogenates of rats treated with NDMA and NDMA + Rsvtrl. ‘M’ is the freshly prepared chicken actomyosin, taken as molecular weight marker. Arrow shows co-stacking of β-actin with α-SMA (Experimental details are given in Materials and Methods). (B) Immunoblot showing the expression of α-SMA in liver homogenates of NDMA and NDMA + Rsvtrl treated animals. Bars showing the relative expression of the α-SMA in the above-mentioned samples. *P < 0.05 and **P < 0.01 vs. control (saline). †Significantly different from NDMA-treated group, P < 0.05.

Conflicts of Interest

The authors declare that there are no conflicts of interest in this article.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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