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Research Article
Hepatoprotective Effect of Diosmin on Iron-induced Liver Damage

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Abstract

Background and Objective: Liver injury is an ascending healthcare challenge. The aim of the present investigation was to elucidate the possible protective effect of diosmin against ferrous sulfate-induced liver injury in adult male albino rats. **Methodology:** Animals were divided into 6 groups; group 1: Normal control (tween 80, p.o., 10 days), group 2: N-acetylcysteine control (300 mg kg^{-1} day^{-1}, p.o., 10 days), group 3: Diosmin control (20 mg kg^{-1} day^{-1}, p.o., 10 days), group 4: Ferrous sulphate (two doses of ferrous sulfate, 30 mg kg^{-1} day^{-1}, i.p., at 9th and 10th day), group 5: N-acetylcysteine (300 mg kg^{-1} day^{-1}, p.o., 10 days) plus ferrous sulphate (two doses of ferrous sulfate, 30 mg kg^{-1} day^{-1}, i.p., at 9th and 10th day) and group 6: Diosmin (20 mg kg^{-1} day^{-1}, p.o., 10 days) plus ferrous sulphate (two doses of ferrous sulfate, 30 mg kg^{-1} day^{-1}, i.p., at 9th and 10th day). On the 11th day, blood and tissue samples were collected. Statistical analysis was carried out using one way analysis of variance ANOVA test followed by Tukey-Kramer multiple comparisons test, with value of $p<0.05$ considered significant. **Results:** Iron-induced liver injury was evidenced by significant increase in hepatocyte membrane damage markers (serum ALT, AST, ALP, GGT, LDH and bilirubin), oxidative and inflammatory markers (hepatic MDA content and NO_x production) and dyslipidemic markers (serum TC and TG) ($p<0.05$). In addition, significant decreases in hepatic GSH content and serum albumin were noted ($p<0.05$). Treatment with diosmin significantly improved hepatocyte membrane damage markers (showing reductions ranging from 24-48%), oxidative and inflammatory markers (showing 34 and 32% reductions regarding MDA and NO_x, respectively), dyslipidemic markers (showing 35 and 39% reductions regarding serum TC and TG, respectively) ($p<0.05$). Histopathological investigation of liver sections, in addition to immunohistochemical investigations of iNOS and eNOS in liver sections, strongly supported biochemical findings. **Conclusion:** Diosmin may have good hepatoprotective effect, mostly through antioxidant and anti-inflammatory potentials. Modulation of NOS expression may have a key role in such protection.

Key words: Diosmin, ferrous sulphate, N-acetylcysteine, hepatotoxicity, eNOS, iNOS, oxidative stress markers

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Iron is a very important polyvalent cation in the body, present in hemoglobin, cytochrome oxidases and other complexes[1]. Physiologically, iron is always present in the body in a bound form, namely with transferrin, ferritin and hemosiderin[2]. If iron load exceeds iron binding capacity, free iron will therefore cause massive oxidative stress, inflammatory reactions and ultimately tissue necrosis. Free iron enhances Reactive Oxygen Species (ROS) formation and lipid peroxidation[3,4]. Iron sulfate has been chosen as a toxic substance for the liver because it is found in many pharmaceutical preparations in the home. There have been many accidents with overdose, either intentionally or accidentally, as happens with children[5].

Ferrous sulphate significantly elevated serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) ($p<0.05$) which indicated hepatocyte membrane damage and release of intracellular hepatic enzymes into circulation. In agreement with Bhattacharya *et al.*[6,7] who stated that 30 mg kg^{-1} ferrous sulphate significantly produced hepatotoxicity in rats evidenced by significant elevations in serum ALT and AST ($p<0.05$). Similarly, Pari *et al.*[8] stated that 30 mg kg^{-1} ferrous sulphate significantly increased serum hepatic markers ($p<0.05$). In particular, serum ALT and AST levels are among the most sensitive indicators of hepatocye membrane damage[9,10].

Diosmin (Diosmetin-7-O-rutinoside), a naturally occurring flavones glycoside readily obtained by dehydrogenation of hesperidin, found abundantly in the pericarp of various citrus[11]. Diosmin has various biological activities including antioxidant activity[12], anti-inflammatory effect[13], anti-diabetic effect[14] and anti-proliferative and anti-cancer activities[12,15]. More-over, diosmin has been found to increase the venous tone and reduce the capillary hyperpermeability, thereby, leading to inhibition of the release of inflammatory mediators[16]. Besides, diosmin has been found to have hepatoprotective effect against many toxic agents[17]. The currently available hepatoprotective drugs such as ursodeoxycholic acid and the other modern hepatoprotective drugs would produce lots of undesirable side effects[18]. Hence, it is of great value to find safe hepatoprotective constituent of herbal medicine, which could alleviate and prevent the progression of hepatic diseases[17].

Although anti-oxidant properties of diosmin have been reported in previous studies[19,20], the effect of diosmin on ferrous sulphate-induced hepatptoxicity has not been investigated. Therefore, the aim of this study was to elucidate the possible protective effect of diosmin against iron-induced hepatotoxicity as compared to N-acetyl cysteine (NAC) as standard treatment in adult male albino rats.

MATERIALS AND METHODS

Materials

Animals: Adult male albino rats, 200-250 g were obtained from the National Research Center, Cairo, Egypt. Animals were housed in plastic cages (28×43×18 cm) and were maintained under standard conditions of temperature (25°C±1) and humidity. Animals were maintained in a clean rodent room and well ventilated with a 12 h light/dark cycle throughout the experimental period in the last winter in Faculty of Pharmacy, Beni-Suef University and were fed standard pellet chow (El-Nasr chemical Co., Cairo, Egypt) with free access to water *ad libitum*.

Drugs

N-acetylcysteine: N-acetylcysteine was purchashed from Sigma-Aldrich (USA) and orally administered[21] in a dose of 300 mg kg^{-1} day^{-1}.

Diosmin: Diosmin was purchashed from Sigma-Aldrich (USA) and orally administered[22] in a dose of 20 mg kg^{-1}.

Chemicals and kits: Serum ALT, AST, albumin and bilirubin reagent kits were obtained from Diamond diagnostics, Egypt. Serum alkaline phosphatase (ALP) kits were obtained from Biodiagnostics, Egypt. Serum gamma glutamyltransferase (GGT) kits were obtained from Analyticon, Germany. Serum lactate dehydrogenase (LDH) kits were obtained from Biosystems, Spain. Serum total cholesterol (TC) and triglycerides (TG) assay kits were obtained from Spinreact, Spain. Disodium hydrogen phosphate and *ortho*- phosphoric acid were obtained from Merck, Germany. Ellman's reagent, ferrous sulphate, glutathione reduced (GSH), malondialdehyde (MDA), N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD), sulfanilamide, sulfosalicylic acid, thiobarbituric acid and vanadium trichloride were obtained from Sigma-Aldrich, USA. The tissue endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS) primary antibodies were obtained from Proteintech, USA. All other chemicals used were of the analytical grade or equal quality.

Experimental design: Rats were divided into 6 groups (each group contains 8 rats) as follows:

Group 1: Normal control (tween 80)
Group 2: N-acetylcysteine (300 mg kg^{-1} day^{-1}) alone
Group 3: Diosmin (20 mg kg^{-1} day^{-1}) alone
Group 4: Hepatotoxicity control group (two doses of ferrous sulphate, 30 mg kg^{-1} day^{-1}, i.p. at 9th and 10th day)
Group 5: N-acetylcysteine plus ferrous sulphate
Group 6: Diosmin plus ferrous sulfate

Group 2 and 3 were given the test agents alone to study the effect of these agents on normal rats.

Treatments were given to rats as oral daily dose for 10 consecutive days. Hepatic injury was induced by i.p. injection of two doses of ferrous sulphate (30 mg kg^{-1}) at 9th and 10th day.

Methods

Induction of liver injury (Ferrous sulphate model): The model was modified from the method described by Bhattacharya *et al.*[6]. The modification was in the number of ferrous sulphate doses where two doses of ferrous sulphate were given instead of one dose in the original model. Treatment agents were given to rats as oral daily dose for 10 consecutive days. Hepatic injury was induced by i.p. injection of two doses of ferrous sulphate (30 mg kg^{-1}) at 9th and 10th day. Animals were anaesthetized by thiopental sodium (75 mg kg^{-1}, i.p.) and blood samples were collected from retro-orbital plexus using heparinized micro-capillary tubes. After that, rats were sacrificed by cervical dislocation to separate liver samples[23].

Manipulation of samples

Blood samples: After collecting blood samples in centrifuge tubes, the tubes were allowed to coagulate at room temperature, then placed in water bath at 37°C for 10 min. Centrifugation at 1000 × g for 20 min was performed. The clear serum was separated and used for analysis of biochemical parameters, including ALT, AST, ALP, LDH, GGT, bilirubin, TC and TG.

Liver samples: After animals were sacrificed, the abdominal cavities were opened and livers were carefully separated, washed with ice-cold saline and the median and left hepatic lobes were used for the preparation of liver homogenate as well as immunological and histopathological examination.

Preparation of liver homogenate: To prepare 20% liver homogenate, 1g of the median lobe was homogenized with 5 volumes of isotonic ice-cooled normal saline using a homogenizer (IKA homogenizer, Model T 25 digital ULTRA-TURRAX, Germany) for the estimation of hepatic MDA and GSH contents as oxidative biomarkers and nitrate/nitrite (NO_x) production as inflammatory biomarker.

Preparation of slides for immunological and histopathological examination: A portion of the liver was kept in well-sealed containers in formalin solution (10%) in normal saline prior to wax embedding, sectioning and staining with haematoxylin and eosin (H and E) for immunological and histological evaluation of liver damage using light microscope attached to a digital camera.

Measurement of biomarkers: Serum ALT and AST were determined according to the method of Reitman and Frankel[24] using commercial kits. Serum ALP was determined according to the method of Belfield and Goldberg[25]. Serum GGT was determined according to the method of Szasz[26] using commercial kits. Serum LDH activity was determined according to the method of Vassault[27] using commercial kits. The GSH was measured in liver homogenate according to the method described by Sedlak and Lindsay[28]. Lipid peroxidation was determined in liver homogenate as thiobarbituric acid reactive substances (TBARS) that were measured as MDA according to the method of Uchiyama and Mihara[29]. The NO_x production in liver tissue was assayed according to the method described by Miranda *et al.*[30]. Serum TG level was assayed according to the method described by Bucolo and David[31] using commercial kits. Serum TC level was assayed according to the method described by Boussekine *et al.*[32] using commercial kits. Serum albumin was determined according to the method of Tietz[33] using commercial kits. Serum total, direct and indirect bilirubin were determined according to the method of Tietz[34] using commercial kits.

Histopathological assessment of liver injury: Autopsy samples were taken from the liver of rats in different groups and fixed in 10% formalin solution in normal saline for 24 h. Washing was done in tap water then serial dilutions of alcohol (methyl, ethyl and absolute ethyl alcohols) were used for dehydration. Specimens were cleared in xylene and embedded in paraffin at 56° in hot air oven for 24 h. Paraffin bees wax tissue blocks were prepared for sectioning at 4 microns thickness by sledge microtome. The obtained tissue sections were collected on glass slides, deparaffinized, stained by hematoxylin and eosin (H and E) stain for routine examination then examination was done through the light electric microscope.

Immunohistochemistry: Immunohistochemistry was performed according to the method of Merz *et al*.[35]. The labelled streptavidin biotin (LSAB) staining method was used in which horseradish peroxidase (HRP), streptividin and two-tiered antibodies were employed to reveal the presence of antigens in a variety of tissues and cell preparations. After the primary antibody has been bound to a target antigen, a secondary antibody that binds specifically to that primary antibody was used. The HRP-labelled streptividin is then bound to the biotinylated secondary antibody and the entire complex is revealed by adding a substrate/chromogen mixture which create an intense color deposit through the activity of the bound enzyme.

Statistical analysis: Data were expressed as the Mean±Standard Error of the Mean (SEM) and comparison between the different treatments was carried out using one-way analysis of variance (ANOVA) test followed by Tukey-Kramer multiple comparisons test, with value of $p<0.05$ considered significant. Statistical analysis was done by the aid of Graph bad prism and Graph pad instant computer software, San Diego, USA.

RESULTS

Effect of 10 days daily oral administration of NAC and diosmin on hepatocyte membrane damage markers in normal rats: The normal group values of serum ALT and AST activities of normal rats were 23.9 and 52.5 U L^{-1}, respectively. The NAC and diosmin did not significantly affect serum ALT or AST activities of rats as compared to normal values. The normal group values of serum ALP, GGT and LDH activities of normal rats were 76.1, 10.9 and 317.2 U L^{-1} respectively. NAC and diosmin did not significantly affect serum ALP, GGT and LDH activities of rats as compared to normal values, the results were given in Table 1.

Effect of 10 days daily oral administration of NAC and diosmin on hepatocyte membrane damage markers in rats with ferrous sulphate-induced hepatotoxicity: The values of serum ALT and AST activities of ferrous sulphate treated rats were 60.8 and 124.8 U L^{-1}, respectively which was significantly higher than the normal group value ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in serum ALT activity to 31.4 and 33.0 U L^{-1}, respectively ($p<0.05$). Pretreatment with N-acetyl cysteine (300 mg kg^{-1} day^{-1}, p.o.) and diosmin (20 mg kg^{-1} day^{-1}, p.o.) significantly reduced ferrous sulphate-induced elevation in serum AST activity to 73.4 and 77.4 U L^{-1}, respectively ($p<0.05$). The values of serum ALP, GGT and LDH activities of ferrous sulphate treated rats were 351.5, 58.8 and 1098.0 U L^{-1}, respectively, which were significantly higher than the normal group value ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in serum ALP activity to 162.5 and 217.5 U L^{-1}, respectively ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in serum GGT activity to 29.4 and 31.0 U L^{-1}, respectively ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in serum LDH activity to 561.7 and 590.5 U L^{-1}, respectively ($p<0.05$); the results were given in Table 2.

Table 1: Effect of 10 days daily oral administration of NAC and diosmin on hepatocyte membrane damage markers in normal rats

Parameters	ALT (U L^{-1})	AST (U L^{-1})	ALP (U L^{-1})	GGT (U L^{-1})	LDH (U L^{-1})
Normal control (2% tween 80, p.o.)	23.9±0.80	52.50±1.37	76.1±5.27	10.9±0.97	317.2±8.85
NAC (300 mg kg^{-1} day^{-1}, p.o.)	20.3±2.05	54.80±3.56	82.9±5.12	13.1±1.08	328.3±19.95
Diosmin (20 mg kg^{-1} day^{-1}, p.o.)	20.3±1.3	51.19±4.26	77.5±4.26	11.8±0.76	323.8±19.7

Each value represents the mean of 6-8 values±SEM. Statistical analysis was carried out using one way ANOVA test followed by Tukey-Kramer multiple comparisons test. NAC: N-acetyl cysteine, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, GGT: Gamma glutamyltransferase, LDH: Lactate dehydrogenase

Table 2: Effect of 10 days daily oral administration of NAC and diosmin on hepatocyte membrane damage markers in rats with ferrous sulphate-induced hepatotoxicity

Parameters	ALT (U L^{-1})	AST (U L^{-1})	ALP (U L^{-1})	GGT (U L^{-1})	LDH (U L^{-1})
Normal control (2% tween 80, p.o.)	23.90±0.80	52.50±1.37	76.1±5.27	10.9±0.97	317.2±8.85
Hepatotoxic control ($FeSO_4$, 30 mg kg^{-1} for 2 days i.p.)	60.80±2.38[a]	124.80±3.24[a]	351.5±10.67[a]	58.8±3.75[a]	1098.0±47.53[a]
NAC+ferrous sulphate	31.40±1.91[ab]	73.40±2.91[ab]	162.5±14.30[ab]	29.4±2.26[ab]	561.7±33.90[ab]
Diosmin+ferrous sulphate	32.96±2.77[ab]	77.43±2.58[ab]	282.0±11.20[abc]	33.0±2.70[ab]	669.6±29.07[ab]

Effect of 10 days daily oral administration of NAC and diosmin on hepatocyte membrane damage markers in rats with ferrous sulphate-induced hepatotoxicity. Each value represents the mean of 6-8 values±SEM. Statistical analysis was carried out using one way ANOVA test followed by Tukey-Kramer multiple comparisons test. a: Significantly different from normal control group at $p<0.05$. b: Significantly different from hepatotoxic control group at $p<0.05$. NAC: N-acetyl cysteine, ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase, GGT: Gamma glutamyltransferase, LDH: Lactate dehydrogenase

Table 3: Effect of 10 days daily oral administration of NAC and diosmin on nitro-oxidative stress markers in normal rats

Parameters	Liver GSH content (mg g^{-1} wet tissue)	Liver MDA content (nmol g^{-1} wet tissue)	Liver NO content (μmol g^{-1} wet tissue)
Normal control (2% tween 80, p.o.)	1.03±0.057	96.09±9.496	280.00±16.810
NAC (300 mg kg^{-1} day^{-1}, p.o.)	1.02±0.066	93.75±7.456	281.20±19.240
Diosmin (20 mg kg^{-1} day^{-1}, p.o.)	1.00±0.051	98.32±9.176	273.10±21.110

Effect of 10 days daily oral administration of NAC and diosmin on nitro-oxidative stress markers in normal rats, each value represents the mean of 6-8 values±SEM. Statistical analysis was carried out using one way ANOVA test followed by Tukey-Kramer multiple comparisons test. NAC: N-acetyl cysteine, GSH: Reduced glutathione, MDA: Malondialdehyde, NO: Nitric oxide

Table 4: Effect of 10 days daily oral administration of NAC and diosmin on nitro-oxidative stress markers in rats with ferrous sulphate-induced hepatotoxicity

Parameters	Liver GSH content (mg g^{-1} wet tissue)	Liver MDA content (nmol g^{-1} wet tissue)	Liver NO content (μmol g^{-1} wet tissue)
Normal control (2% tween 80, p.o.)	1.03±0.057	96.09±9.496	280.00±16.810
Hepatotoxic control ($FeSO_4$, 30 mg kg^{-1} i.p.)	0.31±0.025[a]	230.50±17.480[a]	589.50±20.070[a]
NAC+ferrous sulphate	0.83±0.019[ab]	137.50±12.950[b]	382.00±24.350[ab]
Diosmin+ferrous sulphate	0.84±0.042[abc]	143.90±11.460[ab]	399.20±35.9840[ab]

Effect of 10 days daily oral administration of NAC and diosmin on nitro-oxidative stress markers in rats with ferrous sulphate-induced hepatotoxicity, each value represents the mean of 6-8 values±SEM. Statistical analysis was carried out using one way ANOVA test followed by Tukey-Kramer multiple comparisons test. a: Significantly different from normal control group at p<0.05. b: Significantly different from hepatotoxic control group at p<0.05. c: Significantly different from N-acetyl cycteine treated group at p<0.05. NAC: N-acetyl cysteine, GSH: Glutathione, MDA: Malondialdehyde, NO: Nitric oxide

Table 5: Effect of 10 days daily oral administration of NAC and diosmin on dyslipidemic markers in normal rats

Parameters	Serum TC (mg dL^{-1})	Serum TG (mg dL^{-1})
Normal control (2% tween 80, p.o.)	51.20±2.31	29.80±1.62
NAC (300 mg kg^{-1} day^{-1}, p.o.)	48.40±2.35	30.10±2.03
Diosmin (20 mg kg^{-1} day^{-1}, p.o.)	52.04±3.48	28.81±1.84

Effect of 10 days daily oral administration of NAC and diosmin on dyslipidemic markers in normal rats, each value represents the mean of 6-8 values±SEM. Statistical analysis was carried out using one way ANOVA test followed by Tukey-Kramer multiple comparisons test. NAC: N-acetyl cysteine, TC: Total cholesterol, TG: Triglycerides

Effect of 10 days daily oral administration of NAC and diosmin on nitro-oxidative stress markers in normal rats: The normal group values of liver GSH content, MDA content and NO_x production of normal rats were 1.03 mg g^{-1} wet tissue, 96.09 nmol g^{-1} wet tissue and 280.00 μmol g^{-1} wet tissue, respectively. The NAC and diosmin did not significantly affect liver GSH content, MDA content and NO_x production of rats as compared to normal values, the results were given in Table 3.

Effect of 10 days daily oral administration of NAC and diosmin on nitro-oxidative stress markers in rats with ferrous sulphate-induced hepatotoxicity: The values of liver GSH content, MDA content and NO_x production of ferrous sulphate treated rats were 0.31 mg g^{-1} wet tissue, 230.50 nmol g^{-1} wet tissue and 589.50 μmol g^{-1} wet tissue, respectively, which were significantly different from normal group value ($p<0.05$). Pretreatment with NAC and diosmin significantly elevated ferrous sulphate-induced reduction in liver GSH content to 0.83 and 0.84 mg g^{-1} wet tissue, respectively ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in liver MDA content activity to 137.50 and 143.9 nmol g^{-1} wet tissue, respectively ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in liver NO_x production to 382.00 and 399.20 μmol g^{-1} wet tissue, respectively ($p<0.05$); the results were given in Table 4.

Effect of 10 days daily oral administration of NAC and diosmin on dyslipidemic markers in normal rats: The normal group values of serum TC and TG levels of normal rats were 51.2 and 29.8 mg dL^{-1}, respectively. The NAC and diosmin did not significantly affect serum TC and TG levels of rats as compared to normal values, the results were given in Table 5.

Effect of 10 days daily oral administration of NAC and diosmin on dyslipidemic markers in rats with ferrous sulphate-induced hepatotoxicity: The values of serum TC and TG levels of ferrous sulphate treated rats were 118.5 and 97.8 mg dL^{-1}, respectively, which were significantly higher than the normal group value ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in serum TC level to 75.8 and 77.48 mg dL^{-1}, respectively ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in serum TG level to 59.5 and 60.00 mg dL^{-1}, respectively ($p<0.05$); the results were given in Table 6.

Effect of 10 days daily oral administration of NAC and diosmin on functional markers in normal rats: The normal group value of serum albumin level of normal rats was

5.349 g dL^{-1}. The NAC and diosmin did not significantly affect serum albumin level of rats as compared to normal value. The normal group values of serum total, direct and indirect bilirubin levels of normal rats were 0.304, 0.111 and 0.193 mg dL^{-1} respectively. The NAC and diosmin did not significantly affect serum total, direct and indirect bilirubin levels of rats as compared to normal values, the results were given in Table 7.

Effect of 10 days daily oral administration of NAC and diosmin on functional markers in rats with ferrous sulphate-induced hepatotoxicity: The value of serum albumin level of ferrous sulphate treated rats was 2.748 g dL^{-1} which was significantly lower than the normal group value ($p<0.05$). Pretreatment with NAC and diosmin significantly elevated ferrous sulphate-induced reduction in serum albumin level to 4.538 and 4.552 g dL^{-1} respectively ($p<0.05$). The values of serum total, direct and indirect bilirubin levels of ferrous sulphate-treated rats were 1.847, 0.679 and 1.168 mg dL^{-1}, respectively, which were significantly higher than the normal group value ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in serum total bilirubin level to 0.842 and 0.961 mg dL^{-1}, respectively ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in serum direct bilirubin level to 0.296 and 0.305 mg dL^{-1}, respectively ($p<0.05$). Pretreatment with NAC and diosmin significantly reduced ferrous sulphate-induced elevation in serum indirect bilirubin level to 0.546 and 0.656 mg dL^{-1}, respectively ($p<0.05$); the results were given in Table 8.

Effect of 10 days daily oral administration of NAC and diosmin on liver histopathology in rats with ferrous sulphate-induced hepatotoxicity: Histological sections of hepatic tissue stained with hematoxylin and eosin (H and E) were given in Fig. 1. Histopathological examination of liver sections obtained from normal control group showed normal hepatic architecture with Central Vein (CV) and radiating cords of normal Hepatocytes (H) with central rounded vesicular nuclei and prominent nucleoli. Hepatic cords were separated by blood Sinusoids (S) lined with endothelium and Von-Kupffer cells (white arrow) as shown in Fig. 1a.

On the other hand, liver sections obtained from ferrous sulphate group showed dialted congested Central Vein (CV) with congested blood Sinusoids (S). Massive fatty infiltration of Hepatocytes (H) with some hepatocytes acquired the signet ring appearance (white arrow) as shown in Fig. 1b.

Table 6: Effect of 10 days daily oral administration of NAC and diosmin on dyslipidemic markers in rats with ferrous sulphate-induced hepatotoxicity

Parameters	Serum TC (mg dL^{-1})	Serum TG (mg dL^{-1})
Normal control (2% tween 80, p.o.)	51.20±2.31	29.80±1.62
Hepatotoxic control ($FeSO_4$, 30 mg kg^{-1} i.p.)	118.50±3.81[a]	97.80±4.08[a]
NAC+ferrous sulphate	75.80±2.86[ab]	59.50±2.85[ab]
Diosmin+ferrous sulphate	77.48±4.13[ab]	60.00±2.96[ab]

Effect of 10 days daily oral administration of NAC and diosmin on dyslipidemic markers in rats with ferrous sulphate-induced hepatotoxicity, each value represents the mean of 6-8 values±SEM. Statistical analysis was carried out using one way ANOVA test followed by Tukey-Kramer multiple comparisons test. a: Significantly different from normal control group at $p<0.05$. b: Significantly different from hepatotoxic control group at $p<0.05$. NAC: N-acetyl cysteine, TC: Total cholesterol, TG: Triglycerides

Table 7: Effect of 10 days daily oral administration of NAC and diosmin on Functional markers in normal rats

Parameters	Serum albumin (g dL^{-1})	Serum total bilirubin (mg dL^{-1})	Serum direct bilirubin (mg dL^{-1})	Serum indirect bilirubin (mg dL^{-1})
Normal control (2% tween 80, p.o.)	5.349±0.2351	0.304±0.0251	0.111±0.0098	0.193±0.0156
NAC (300 mg kg^{-1} day^{-1}, p.o.)	4.958±0.2997	0.310±0.0266	0.106±0.0116	0.204±0.0165
Diosmin (20 mg kg^{-1} day^{-1}, p.o.)	5.015±0.3003	0.288±0.0267	0.0936±0.0089	0.194±0.0181

Each value represents the mean of 6-8 values±SEM. Statistical analysis was carried out using one way ANOVA test followed by Tukey-Kramer multiple comparisons test. NAC: N-acetyl cysteine

Table 8: Effect of 10 days daily oral administration of NAC and diosmin on functional markers in rats with ferrous sulphate-induced hepatotoxicity

Parameters	Serum albumin (g dL^{-1})	Serum total bilirubin (mg dL^{-1})	Serum direct bilirubin (mg dL^{-1})	Serum indirect bilirubin (mg dL^{-1})
Normal control (2% tween 80, p.o.)	5.349±0.2351	0.304±0.0251	0.111±0.0098	0.193±0.0156
Hepatotoxic control ($FeSO_4$, 30 mg kg^{-1} i.p.)	2.748±0.2529[a]	1.847±0.0739[a]	0.679±0.0310[a]	1.168±0.0444[a]
NAC+ferrous sulphate	4.538±0.3603[b]	0.842±0.0478[ab]	0.296±0.0191[ab]	0.546±0.0291[ab]
Diosmin+ferrous sulphate	4.552±0.4165[b]	1.105±0.0679[ab]	0.305±0.0224[ab]	0.656±0.0627[ab]

Effect of 10 days daily oral administration of NAC and diosmin on functional markers in rats with ferrous sulphate-induced hepatotoxicity, each value represents the mean of 6-8 values±SEM. Statistical analysis was carried out using one way ANOVA test followed by Tukey-Kramer multiple comparisons test. a: Significantly different from normal control group at $p<0.05$. b: Significantly different from hepatotoxic control group at $p<0.05$, NAC: N-acetyl cysteine

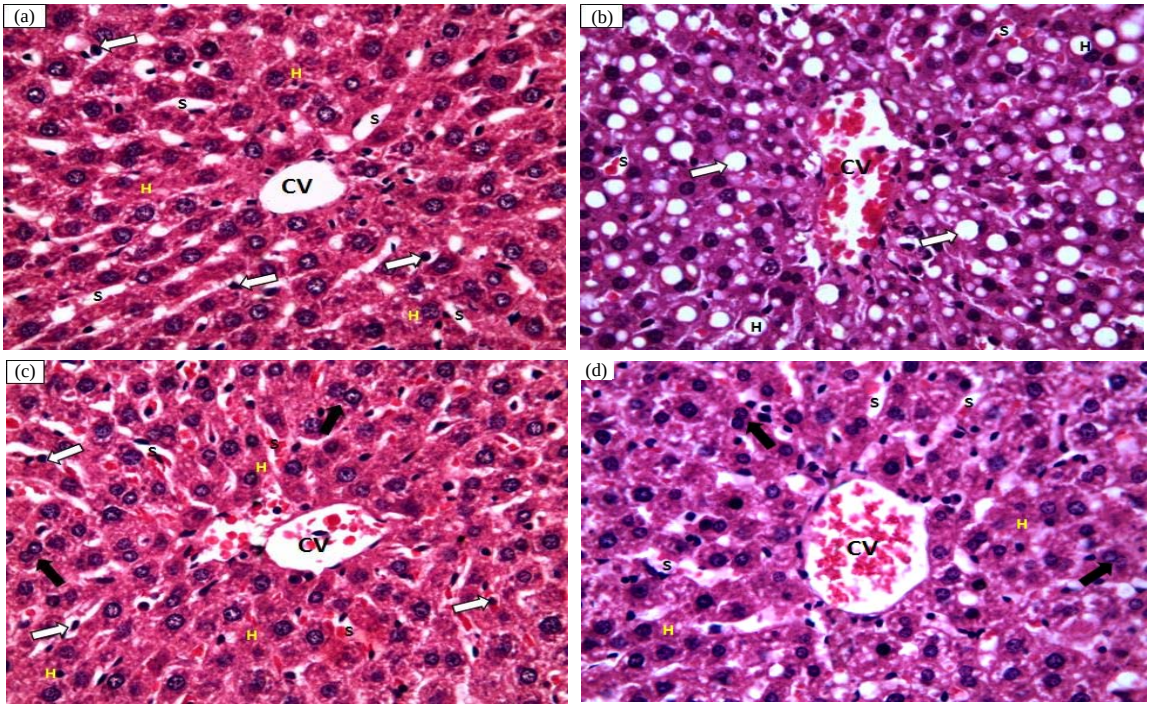

Fig. 1(a-d): A photomicrograph of liver section obtained from different groups (H and E; 400x), (a) Normal control group, (b) Ferrous sulphate group, (c) N-acetylcysteine plus ferrous sulphate group and (d) Diosmin plus ferrous sulphate group. CV: Central vein, H: Normal hepatocytes, S: Blood sinusoids, white arrow: Von-Kupffer cells, black arrow: Binucleated cells

Animal treated with NAC plus ferrous sulphate showed congested Central Vein (CV). Normal Hepatocytes (H) are separated by slightly dilated congested blood Sinusoids (S) with activated Von-Kupffer cells (white arrow). binucleated cells (black arrow) can be seen as shown in Fig. 1c.

Treatment of rats with diosmin plus ferrous sulphate showed congested Central Vein (CV). Normal Hepatocytes (H) are separated by slightly dilated congested blood Sinusoids (S) with activated Von-Kupffer cells (white arrow). Binucleated cells (black arrow) can be seen as shown in Fig. 1d.

Effect of 10 days daily oral administration of NAC and diosmin on immunohistochemical staining in rats with ferrous sulphate-induced hepatotoxicity

Inducible nitric oxide synthase (iNOS): Normal control group showed weak immunoreactivity to iNOS which appeared as light brown colour (white arrow) as shown in Fig. 2a. Ferrous sulphate group showed strong immunoreactivity to iNOS which appeared as brown colour (white arrow) with some areas of intense immunoreactivity (yellow arrow) as shown in Fig. 2b. The NAC plus ferrous sulphate group showed mild immunoreactivity to iNOS which appeared as brown colour (white arrow) as shown in Fig. 2c. Diosmin plus ferrous sulphate group showed mild immunoreactivity to iNOS which appeared as brown colour (white arrow) as shown in Fig. 2d.

Endothelial nitric oxide synthase (eNOS): Normal control group showed strong immunoreactivity to eNOS which appeared as brown colour (white arrow) as shown in Fig. 3a. Ferrous sulphate group showed weak to negative immunoreactivity to eNOS which appeared as brown colour (white arrow) as shown in Fig. 3b. The NAC plus ferrous sulphate group showed strong immunoreactivity to eNOS which appeared as brown colour (white arrow) but not as normal control group as shown in Fig. 3c. Diosmin plus ferrous sulphate group showed strong immunoreactivity to eNOS which appeared as brown colour (white arrow) as shown in Fig. 3d.

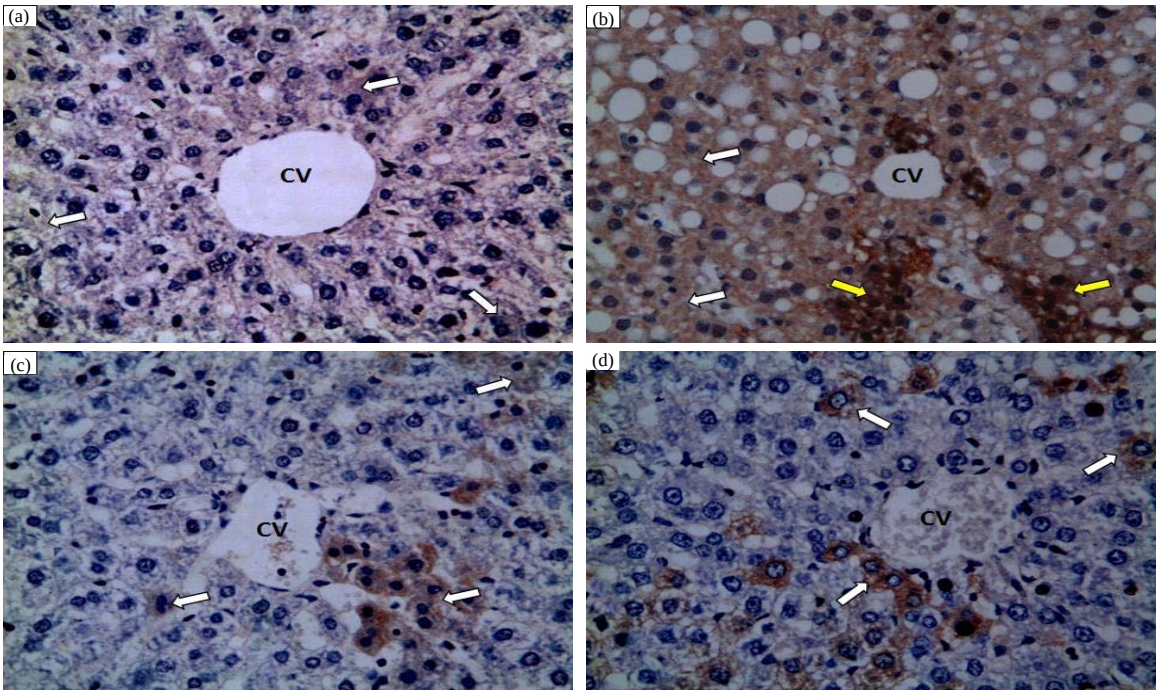

Fig. 2(a-d): A photomicrograph of liver section obtained from different groups (iNOS immunohistochemical stain; 400x), (a) Normal control group, (b) Ferrous sulphate group, (c) N-acetylcysteine plus ferrous sulphate group and (d) Diosmin plus ferrous sulphate group. CV: Central vein, white arrow: Brown colour, yellow arrow: Areas of intense immunoreactivity

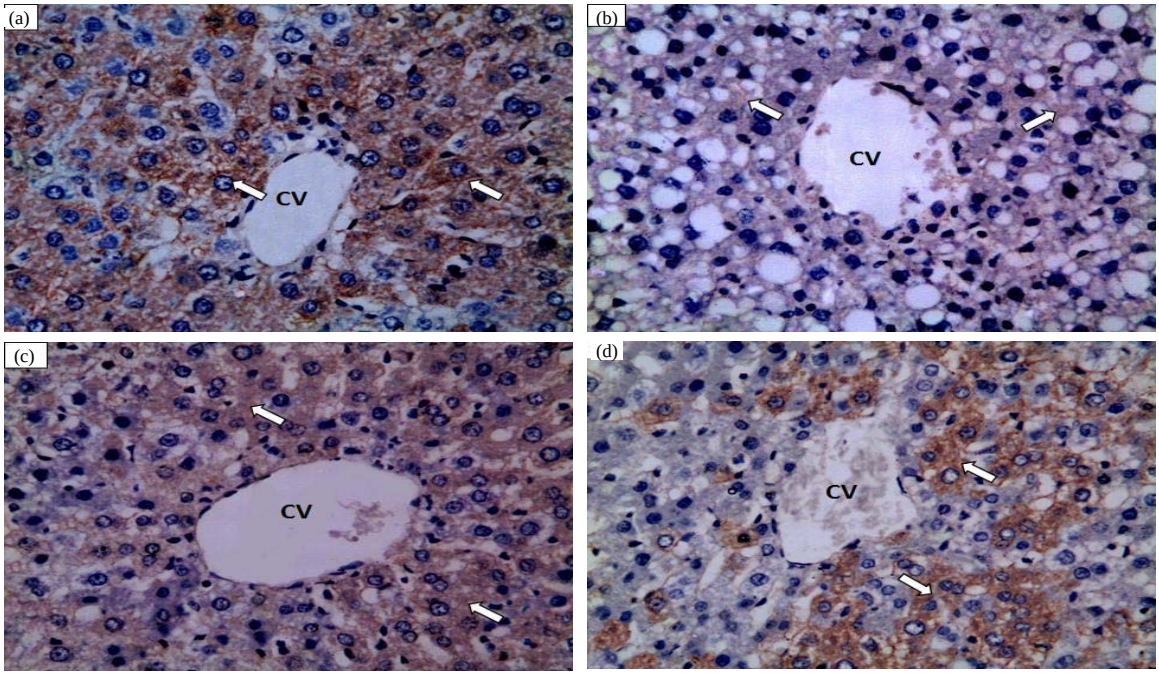

Fig. 3(a-d): A photomicrograph of liver section obtained from different groups (eNOS immunohistochemical stain; 400x), (a) Normal control group, (b) Ferrous sulphate group, (c) N-acetylcysteine plus ferrous sulphate group and (d) Diosmin plus ferrous sulphate group. CV: Central vein, white arrow: Brown colour

DISCUSSION

In this study, diosmin effect was studied in hepatotoxic rats. Hepatotoxicity was induced by ferrous sulphate which increased hepatic markers. Ferrous sulphate significantly elevated serum activities of ALT, AST, ALP, GGT, LDH and bilirubin ($p<0.05$) which indicated hepatocyte membrane damage and release of intracellular hepatic enzymes into circulation. Increase in serum level of ALP is due to increased synthesis, in presence of increasing biliary pressure[36]. The LDH is also an intracellular enzyme, whose release indicates cell damage[37]. Serum GGT has been used as indicator of liver dysfunction and in the presence of iron, the products of the GGT reaction may themselves increased the production of free radical[38].

Findings of the present study revealed that ferrous sulphate caused significant increase in liver MDA and NO_x production as compared to normal control group ($p<0.05$) and significant reduction in liver GSH content ($p<0.05$). The results of the present study are in accordance with those obtained by other investigators[8,39]. The target of iron toxicity is the mitochondrion, where iron overload causes oxidative mitochondrial membrane damage and damage of enzymes of the tri-carboxylic acid cycle[40]. Iron has redox properties and consequently it catalyzes a number of functions in the cells[41]. However, these redox properties render iron able to generate ROS and destroy liver cells[42]. The alterations in structure and function of cells caused by overload of iron seem to be related to free radical-mediated cell components damage. The chemical structure of iron and its ability to drive one-electron reactions makes it a major player in the production of free radicals in the biological systems[43].

Iron overload was found to induce nitric oxide expression leading to increased nitric oxide production which form peroxynitrite (in combination with superoxide anions) which is dangerous mediator of lipid peroxidation[44]. It is clear from the immunohistochemical study that iron administration caused significant increase in iNOS expression ($p<0.05$), potentiating oxido-nitrosative stress and inflammatory outcome.

Concerning the results of the present study, too, ferrous sulphate showed significant elevation of serum levels of TG and TC ($p<0.05$). Similar results were obtained by Pari *et al.*[8] who reported that serum cholesterol and triglycerides levels were significantly higher in rats after administration of 30 mg kg^{-1} ferrous sulphate when compared to normal group ($p<0.05$). Ferrous sulphate causes disturbances of mitochondrial function, which leads to inhibition of β-oxidation and accumulation of serum free fatty acids and triglycerides. Serum cholesterol also increased due to changes in the expression of the gene of the liver enzyme HMG-COA reductase[45].

Findings of the present study revealed that iron overload significantly reduced serum albumin level as compared to normal control group ($p<0.05$). The results are in accordance with those obtained by Kaur *et al.*[46] who reported that serum albumin level was significantly lower in mice after i.p. administration of 9 mg kg^{-1} ferric nitrilotriacetate when compared to normal group ($p<0.05$). Albumin is the most important protein synthesized in the liver and its concentration is a good indicator of liver functional integrity[47].

Findings of the present study revealed that iron overload significantly increased serum bilirubin level as compared to normal control group ($p<0.05$). The results of the present study are in accordance with those obtained by Pari *et al.*[8] who reported that serum bilirubin level was significantly higher in rats after administration of 30 mg kg^{-1} ferrous sulphate as compared to normal group ($p<0.05$). Bilirubin is the excretory end product of heme degradation. It is conjugated in the liver with glucuronic acid and then excreted into the bile. Elevated plasma concentration of free (indirect) bilirubin is a marker for serious liver injury[47].

The current data showed that diosmin significantly suppressed ferrous sulphate-induced increase in serum activities of ALT, AST, ALP, GGT and LDH ($p<0.05$). The results are in accordance with those obtained by Tahir *et al.*[22] who stated that diosmin protect liver cells against ethanol induced hepatotoxicity in rats. Diosmin restored the changes in serum activities of ALT, AST, ALP, GGT and LDH due to its antioxidant effect, thereby protecting membrane permeability[22]. Findings of the present study revealed that diosmin caused significant decrease in liver MDA and NO_x production as compared to ferrous sulphate control group ($p<0.05$), coupled with significant increase in liver GSH content ($p<0.05$). The results are in accordance with those obtained by other investigators, where Abdel-Salam *et al.*[17] stated that diosmin counteracted lipopolysaccharide-induced oxidative stress due to its antioxidant effect.

Concerning the results, diosmin showed significant decrease of serum levels of TG and TC as compared to ferrous sulphate group ($p<0.05$). The results are in accordance with those obtained by Queenthy and John *et al.*[48] who studied the effect of diosmin on serum triglycerides in isoproterenol induced myocardial infarcted rats. The effect of diosmin is

related to inhibition of both hepatic HMG CoA reductase and acyl CoA cholesterol acyl-transferase (ACAT) activities[48].

Diosmin corrected the serum level of albumin due to its ability to protect the hepatocytes against toxic injuries and restore the normal function of the liver[22]. Findings of the present study revealed that diosmin significantly decreased serum bilirubin level as compared to ferrous sulphate control group ($p<0.05$). the results are in accordance with those obtained by Tahir *et al.*[22] who studied the effect of diosmin in ethanol-induced hepatotoxicity in rats. The elevated serum level of bilirubin is an indicator of hepatocyte injury[49]. Diosmin protect liver cells against toxicity due to its antioxidant effect, thereby protecting membrane permeability and decreasing serum bilirubin[22].

The immunohistochemical study results showed that suppression of iNOS expression may be, at least partly, a logic mechanism of antioxidant and anti-inflammatory effects of diosmin. NOx overproduction causes oxido-nitrosative stress since peroxynitrite, the product of interaction between nitrite and hydrogen peroxide, is a highly damaging agent[50]. The eNOS releases physiological beneficial levels of nitric oxide[51], while iNOS is up-regulated in pathological conditions, causing deleterious effects[52]. Interestingly, diosmin in the current study ameliorated harmful iNOS expression without ameliorating eNOS expression.

CONCLUSION

It is concluded that ferrous sulphate produced severe hepatotoxicity in rats, where oxidative stress play an important role as evidenced by increased liver contents of MDA, NO and depletion of GSH. Diosmin is actually a good hepatoprotective agent preserving membrane integrity, ameliorating oxido-nitrosative stress and correcting dyslipidemia. Suppression of iNOS expression may be a logic mechanism involved in such protection. These results are promising for further clinical trials.

SIGNIFICANCE STATEMENTS

This study discovered that diosmin can be a promising agent for clinical use as hepatoprotection against ferrous sulphate-induced hepatotoxicity. Their effects were similar to that of N-acetyl cysteine. This study also signifies that the most likely proposed mechanism for the hepatic protection conferred by this drug is related to its antioxidant properties and its ability to decrease inducible reperfusion nitric oxide and increase ischemic nitric oxide.

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