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N-acetylcysteine attenuates dimethylnitrosamine induced oxidative stress in rats

Priya Sathish, Vijayalakshmi Paramasivan, Vivekanandan Palani, Karthikeyan Sivanesan *

Department of Pharmacology and Environmental Toxicology, Dr. A.L.M. Postgraduate Institute of Basic Medical Sciences, University of Madras, Taramani, Chennai - 600 113, India

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ABSTRACT

Oxidative stress has been implicated in the pathogenesis and progression of various hepatic disorders and hence screening for a good hepatoprotective and antioxidant agent is the need of the hour. The present study was aimed to investigate the hepatoprotective and antioxidant property of *N*-acetylcysteine (NAC) against dimethylnitrosamine (DMN) induced oxidative stress and hepatocellular damage in male Wistar albino rats. Administration of single dose of DMN (5 mg/kg b.w.; i.p.) resulted in significant elevation in the levels of serum aspartate transaminase and alanine transaminase, indicating hepatocellular damage. Oxidative stress induced by DMN treatment was confirmed by an elevation in the status of lipid peroxidation (LPO) and reduction in the activities of enzymic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase and glutathione-S-transferase and in the levels of non-enzymic antioxidants, reduced glutathione, vitamin-C and vitamin-E in the liver tissue. DMN induced oxidative stress and hepatocellular membrane instability was further substantiated by a decline in the status of the membrane bound ATPases in the liver tissue. Post-treatment with NAC (50 mg/kg b.w.; p.o.) for 7 days effectively protected against the DMN induced insult to liver by preventing the elevation in the status of the serum marker enzymes and LPO, and restoring the activities of both the enzymic and non-enzymic antioxidants and membrane bound ATPases towards normalcy. These results demonstrate that NAC acts as a good hepatoprotective and antioxidant agent in attenuating DMN induced oxidative stress and hepatocellular damage.

1. Introduction

Dimethylnitrosamine (DMN) is a well established hepatotoxin, carcinogen and mutagen in experimental animals. DMN poses public health concerns because humans are exposed to it via food stuffs such as fish, cured meats, cheese, bacon, tobacco smoke, chewing tobacco, beer, use of toiletries, cosmetics and rubber products (ATSDR (Agency for Toxic Substances and Disease Registry), 1989). DMN exerts its hepatotoxic and carcinogenic effects through metabolic activation by CYP2E1 (George et al., 2001). The hepatotoxic metabolites of DMN interact with the tissue macromolecules leading to carcinogenicity and hepatotoxicity. Oxidative stress is said to play a major role in the onset of DMN induced hepatocellular damage through the generation of reactive oxygen species (Hermanns et al., 1994). Hence it is essential to find out a potential hepatoprotective and antioxidant agent which is effective in protecting the liver against DMN induced oxidative stress and hepatotoxicity. Numerous studies are available in literature on the hepatoprotective and antioxidant effects of plant extracts *Lonicera japonica* Thunb. (Caprifoliaceae) (Sun et al., 2010) and compounds such as kolaviron (Farombi et al., 2009) and pirfenidone (Tada et al., 2001) against DMN induced hepatotoxicity. In light of these studies, it was decided to evaluate the hepatoprotective and antioxidant properties of *N*-acetylcysteine (NAC) against DMN induced oxidative stress and hepatotoxicity.

N-acetylcysteine (NAC) is a thiol containing antioxidant, which has been in clinical use since 1960s as a mucolytic agent in the treatment of various pulmonary disorders (Grassi and Morandini, 1976). It is considered as the drug of choice for the treatment of acetaminophen poisoning (Lauterburg et al., 1983). NAC exerts its antioxidant action by facilitating reduced glutathione biosynthesis and scavenging the reactive oxygen species formed during oxidative stress (Ocal et al., 2004). NAC has been shown to offer protection against liver injury induced by various chemical hepatotoxins such as ethanol (Ronis et al., 2005), methanol (Raza et al., 2003) and CCl_4 (Wong et al., 2003; Galicia et al., 2010). In addition to its hepatoprotective action, NAC is also widely used as an antiangiogenic (Albini et al., 2001), antifibrotic (Filho et al., 2008), neuroprotective (Eskiocak et al., 2008), renoprotective (Heyman et al., 2003) and as a chelating agent in the treatment of heavy metal poisoning (Modi et al., 2006). In the present study we investigated the hepatoprotective and antioxidant effects of NAC against DMN induced oxidative stress and hepatotoxicity in male Wistar albino rats.

2. Materials and methods

2.1. Animals

Male Wistar albino rats weighing 200 to 220 g were used for this study. The animals were housed in polypropylene cages under

* Corresponding author. Tel.: +91 44 2454 7144; fax: +91 44 2454 0709.
E-mail address: pripharm2010@gmail.com (K. Sivanesan).

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controlled environmental conditions (temp: 27 ± 2 °C; relative humidity: 50–70%; 12 h light/dark cycle) and were provided standard pellet feed and water ad libitum. The animal experiments were performed after getting prior approval from the Institutional Animal Ethical Committee (IAEC), governed by guidelines prescribed by CPCSEA.

2.2. Chemicals

Dimethylnitrosamine (DMN) and malondialdehyde (MDA) were purchased from M/s. Sigma Aldrich Chemicals, Pvt. Ltd., USA. *N*-acetylcysteine (NAC) was purchased from M/s. SISCO Research Laboratory, Chennai, India. All the other chemicals used were of analar grade (AR) and they were purchased locally.

2.3. Experimental design

The rats were divided into four groups with 6 animals in each group. Group-I rats served as control and were treated with saline on day zero and were left without any treatment for 7 days. Group-II and III rats were administered a single dose of DMN (5 mg/kg b.w.; i.p.) on day zero. Thereafter, while Group-II rats were left without any treatment, Group-III rats were treated with NAC (50 mg/kg b.w.; p.o.) daily for 7 days. Group-IV rats received NAC alone (50 mg/kg b.w.; p.o.) daily for 7 days. The dose of DMN was fixed as 5 mg/kg based on previous published reports (Nicolini et al., 1976). NAC dosage was selected based on the earlier reports of Kalaiselvi et al. (2005).

At the end of the experimental period, all the animals were subjected to mild ether anesthesia and blood was collected from retro orbital plexus and serum was separated by centrifugation at 2500 rpm for 15 min. The animals were sacrificed by cervical decapitation and the liver tissue was excised quickly and washed in ice cold saline and blotted to dryness. A 1% liver tissue homogenate was prepared in Tris–HCl buffer (0.1 M; pH 7.4), centrifuged and clear supernatants separated were preserved at (12 to 15 °C) in vials for further biochemical analysis. A piece of liver tissue was also sectioned out and placed immediately in phosphate buffered formal saline and stained with hematoxylin–eosin for histopathological analysis.

The activities of aspartate and alanine transaminases (AST and ALT) in serum and liver tissue were estimated according to the method of Reitman and Frankel (1957). Lipid peroxidation (LPO) in the liver tissue homogenate was determined as described by Ohkawa et al. (1979). The activities of the enzymic antioxidants superoxide dismutase (SOD) and catalase (CAT) were assayed according to the methods of Marklund and Marklund (1974) and Sinha (1972) respectively. The reduced glutathione (GSH) content in the liver tissue was determined according to the method of Ellman (1959) with little modification (Beutler et al., 1963). The activity of glutathione-S-transferase (GST) was estimated in the liver tissue by the method described by Habig et al. (1974). The activity of glutathione peroxidase (GPx) was investigated based on the procedures described by Rotruck et al. (1973) and Beutler et al. (1963). The activity of glutathione reductase (GR) was estimated according to the method of Mize and Langdon (1962). The vitamin-C content in the liver tissue was determined according to the method of Omaye et al. (1979) and vitamin-E according to Varley et al. (1976). The activity of Na^+/K^+ ATPase was estimated in the liver tissue according to the method detailed by Bonting (1970), Mg^{2+} ATPase activity was estimated in the liver tissue by the method of Ohnishi et al. (1982) and Ca^{2+} ATPase activity was estimated according to the method described by Hjerten and Pan (1983) and the phosphate liberated was determined according to the procedure detailed by Fiske and Subbarow (1925). Protein was estimated based on the method described by Lowry et al. (1951).

2.4. Statistical analysis

The data was subjected to one-way ANOVA and Tukey's multiple comparison test was done to evaluate the significance of difference in means between various treatment groups using SPSS statistical package (Version: 7.5). Values are presented as mean ± S.D. and P value < 0.05 was considered significant.

3. Results

3.1. Effect of NAC on DMN induced alterations in the activities of AST and ALT and histopathology of liver tissue of rats

The activities of AST and ALT in serum and liver tissue and histopathology of liver tissue of control and DMN treated rats are presented in Fig. 1A, B, and C, respectively. DMN treatment (Group-II) produced a significant 3–4 fold increase in the activities of AST and ALT in serum when compared to control (Group-I). In contrast to this increase, there was a significant decrease in the activities of these marker enzymes in the liver tissue upon DMN administration. Post-treatment with NAC (Group-III) for 7 days significantly restored the altered activities of these marker enzymes both in serum and liver tissue towards normalcy. Histopathology of the liver tissue of rats treated DMN (Group-II) shows extensive sinusoidal dilatation around portal triad area. The portal triaditis is marked by disruption of basement membrane and abundant infiltration of neutrophils around portal triad area. The chromatin is condensed and more number of hepatocytes shows pre-malignant changes, indicating onset of carcinogenicity. The sinusoidal space is dilated and infiltrated by neutrophils. Histopathology of DMN + NAC (Group-III) treated rats shows recovery of the liver tissue. Few hepatocytes still show hyperchromatic nucleus with mild portal triaditis and infiltration of neutrophils in the dilated sinusoidal space. NAC alone (Group-IV) shows normal histological architecture of the liver tissue and was comparable to saline treated control (Group-I).

3.2. Effect of NAC on DMN induced alterations in the levels of non-enzymic antioxidants (LPO, GSH, vitamin-C and vitamin-E) in the liver tissue of rats

The changes in the levels of LPO, GSH, vitamin-C and vitamin-E in the liver tissue of rats are presented in Fig. 2A, B, C, and D, respectively. DMN (Group-II) administration caused a significant 3-fold elevation in the levels of LPO and a 40–50% fall in the levels of GSH, vitamin-C and vitamin-E as compared to control (Group-I). Post-treatment with NAC (Group-III) for 7 days after DMN administration decreased the levels of these non-enzymic antioxidants when compared with DMN alone (Group-II) treated rats.

3.3. Effect of NAC on DMN induced alterations in the activities of enzymic antioxidants (SOD, CAT, GPx, GST and GR) in liver tissue of rats

Fig. 3(A, B, C, D, E) shows the activities of enzymic antioxidants (SOD, CAT, GPx, GST and GR) in the liver tissue of rats. A significant fall in the activities of all the above enzymic antioxidants was observed after DMN administration (Group-II). NAC post-treatment (Group-III) for 7 days, significantly prevented the fall in their activities and restored them back towards normalcy.

3.4. Effect of NAC on DMN induced alterations in the activities of the membrane bound ATPases (Na^+/K^+, Mg^{2+} and Ca^{2+} ATPases) in liver tissue of rats

Fig. 4 illustrates the activities of the membrane bound ATPases (Na^+/K^+, Mg^{2+} and Ca^{2+} ATPases) in the liver tissue of rats. DMN treatment (Group-II) caused a significant decrease in the activities of Na^+/K^+, Mg^{2+} and Ca^{2+} ATPases as compared to control (Group-I). NAC post-treatment (Group-III) significantly prevented the above decrease in the activities of all ATPases induced by DMN treatment. NAC alone (Group-IV) administration did not produce any significant

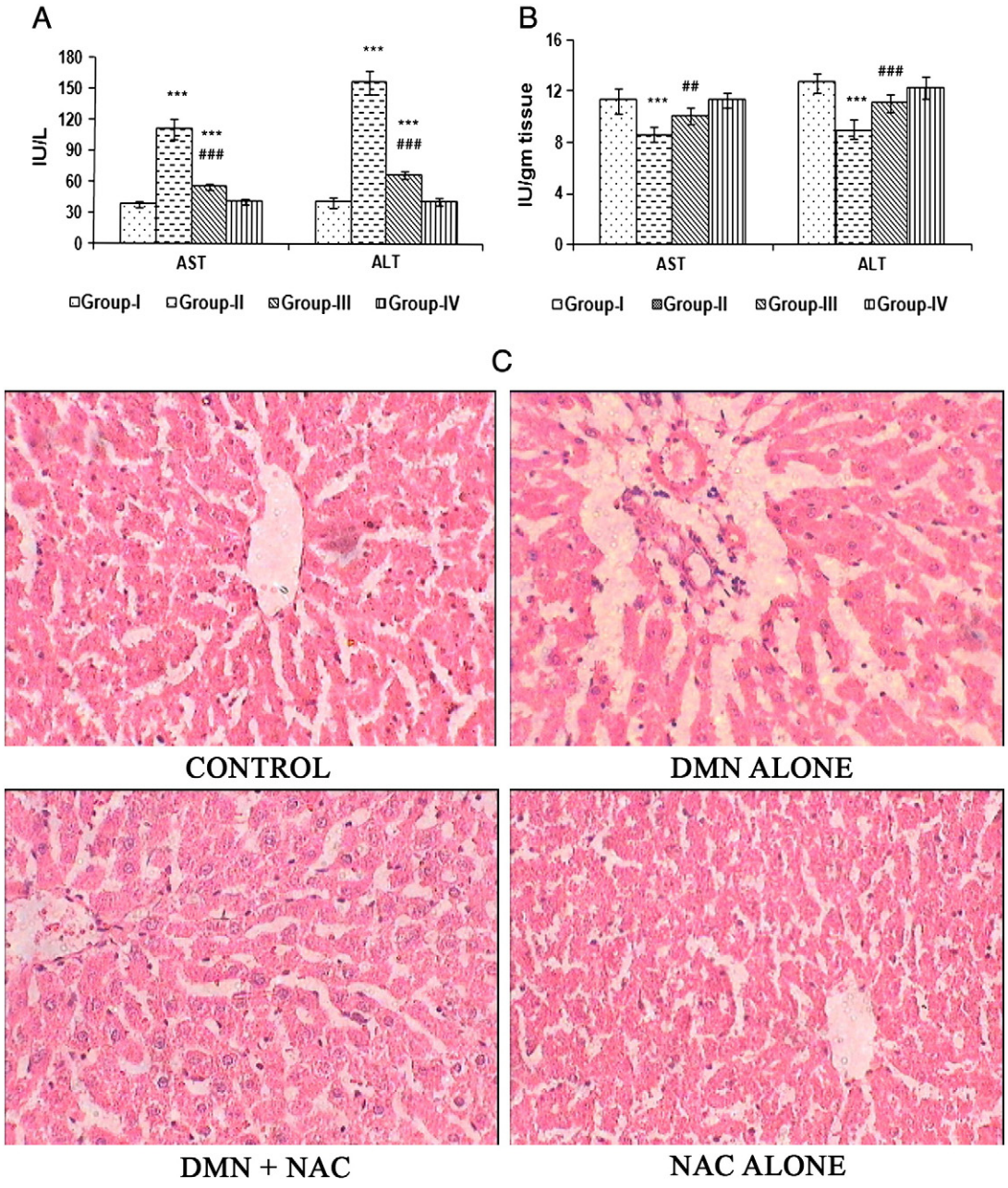

Fig. 1. Effect of NAC on DMN induced changes in AST, ALT and histopathology of liver tissue of rats. (A) Status of AST and ALT in serum. (B) Status of AST and ALT in liver tissue. Values are mean ± S.D. of six rats. ***P<0.001 Vs Group-I; ##P<0.01; ###P<0.001 Vs Group-II. (C) Histopathology of liver tissue of control and experimental animals (×40).

change in the status of any of the above parameters investigated in serum and liver tissue and were similar to that of control (Group-I).

4. Discussion

Dimethylnitrosamine (DMN), a well established hepatotoxin, carcinogen and mutagen has been reported to induce oxidative stress and hepatocellular damage in rats (Hermanns et al., 1994). The reactive oxygen species generated during the metabolism of DMN in the liver has been suggested to be the cause for the induction of oxidative stress. In this investigation, DMN induced hepatocellular damage is clearly evidenced by an elevation in the levels of aspartate transaminase (AST) and alanine transaminase (ALT) in serum, accompanied by a subsequent fall in their levels in the liver tissue. Elevation in the levels of serum transaminases observed in this study might be due to the release of these enzymes from the cytoplasm into the blood circulation rapidly after rupture of the plasma membrane and cellular damage caused by DMN treatment which is further substantiated by a simultaneous fall in their levels in the liver tissue. The reactive oxygen species and free radicals generated during the hepatic metabolism of DMN might have caused the hepatocellular damage and leakage of the cellular enzymes. Similar elevation in the status of serum AST and ALT during DMN administration has been reported by Hsu et al. (2007) and Wang et al. (2010). *N*-acetylcysteine (NAC) post-treatment significantly prevented the alterations in the status of these marker enzymes by maintaining the plasma membrane integrity which indicates its hepatoprotective properties.

Oxidative stress in a cell is said to occur when the antioxidant defense system is overwhelmed by the production of reactive oxygen species and free radicals (Nencini et al., 2007). Lipid peroxidation (LPO) as well as altered levels of some endogenous scavengers is taken as direct *in vivo* reliable indices for free radical generation and oxidative stress (EL-Khatib et al., 2001). Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) act mutually to

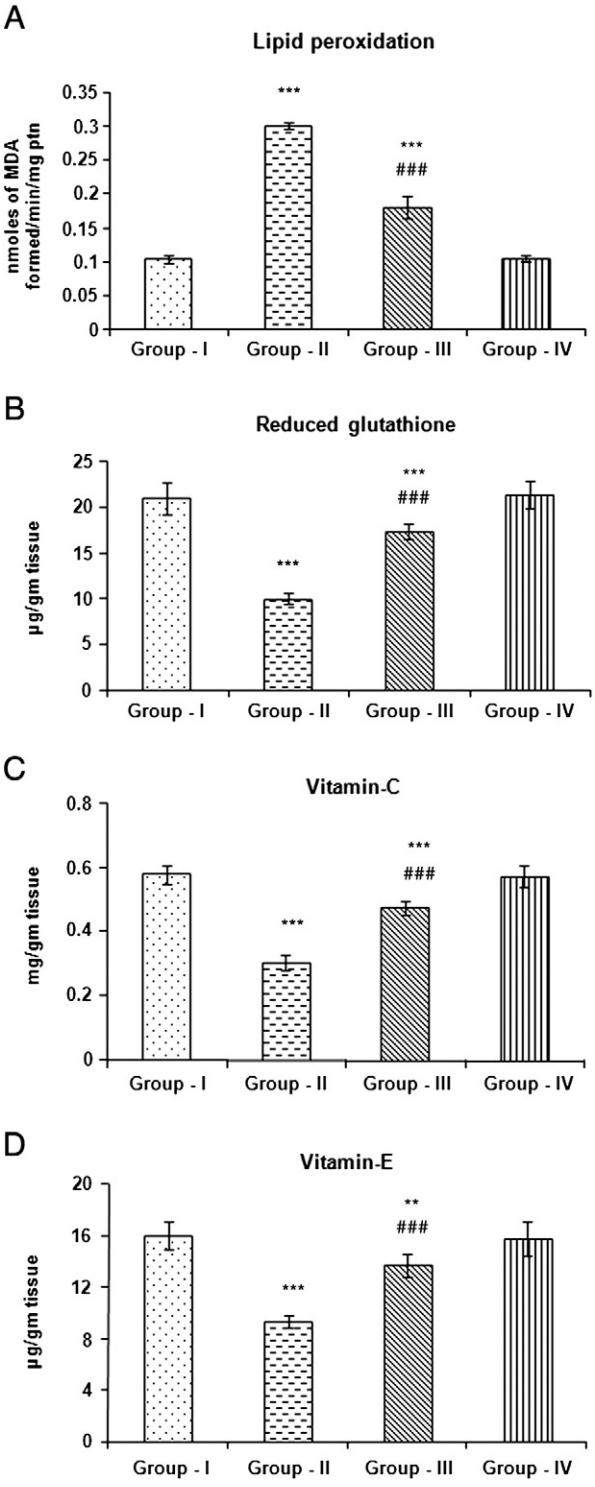

Fig. 2. Effect of NAC on DMN induced changes in the status of non-enzymic antioxidants in the liver tissue of rats. Values are mean ± S.D. of six rats. **P<0.01; ***P<0.001 Vs Group-I; ###P<0.001 Vs Group-II.

constitute the enzymic antioxidant defense mechanism against reactive oxygen species (Bhattacharjee and Sil, 2006). A decrease in the activity of SOD, CAT and GPx observed in this study could possibly be due to their over utilization towards suppression of reactive oxygen species and subsequent free radicals that is liberated during

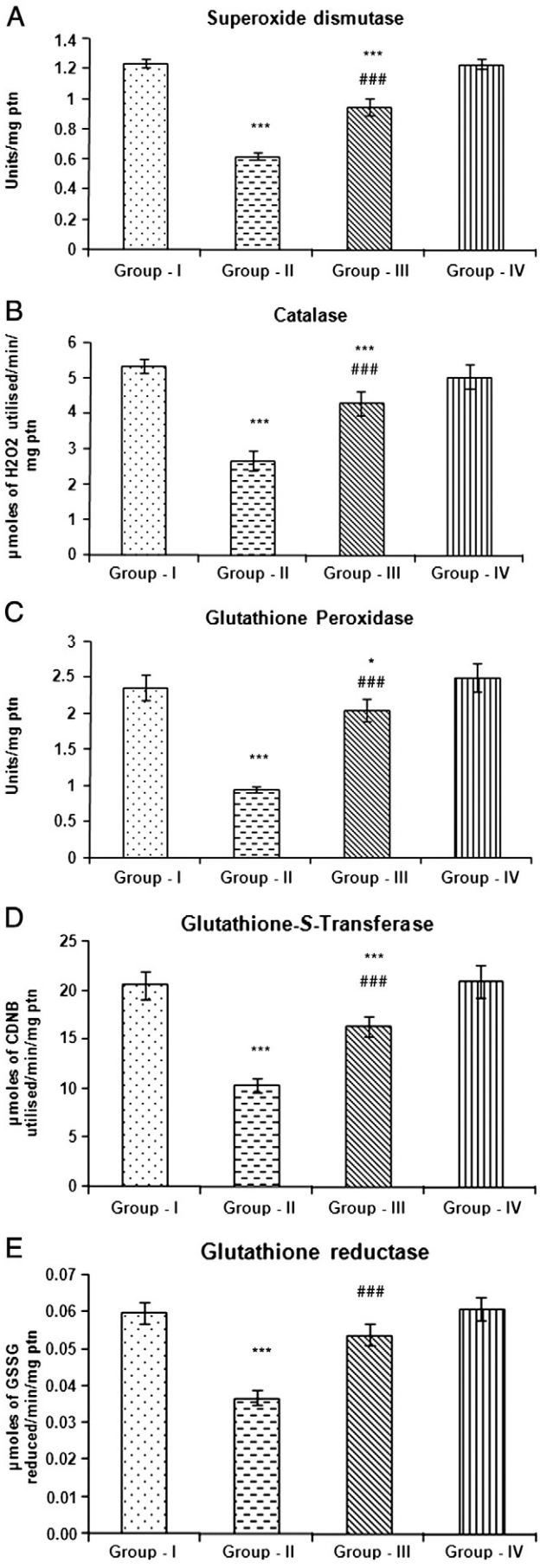

Fig. 3. Effect of NAC on DMN induced changes in the activities of enzymic antioxidants in the liver tissue of rats. Values are mean ± S.D. of six rats. *P<0.01; ***P<0.001 Vs Group-I; ###P<0.001 Vs Group-II.

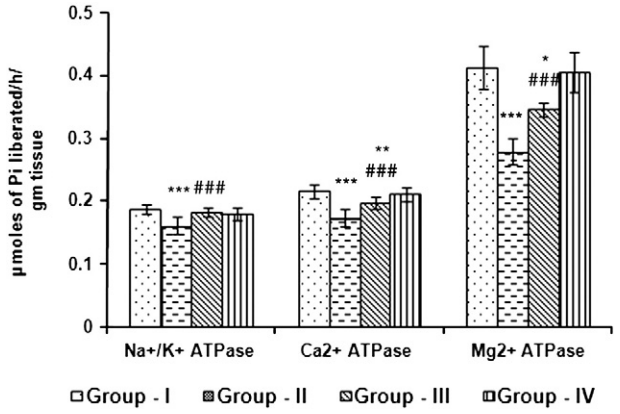

Fig. 4. Effect of NAC on DMN induced changes in the activities of the membrane bound ATPases in the liver tissue of rats. Values are mean ± S.D. of six rats. *P<0.05; **P<0.01; ***P<0.001 Vs Group-I; ###P<0.001 Vs Group-II.

metabolism of DMN. Studies conducted by Wang et al. (2010) have also reported similar reduction in the status of these enzymic antioxidants on DMN administration in rats. Increase in LPO levels and induction of oxidative stress have been reported in DMN induced hepatotoxicity (George, 2003; Farombi et al., 2009) and our results are in accordance with these reports. NAC post-treatment effectively counteracted the DMN induced oxidative stress which is evident from the restoration of the elevated LPO levels and the status of the diminished antioxidant enzymes, indicating its potentiality to act as a powerful antioxidant and free radical scavenger by preventing the peroxidative damage caused by DMN. These findings are supported by the investigations conducted by Ozaras et al. (2003) on the ameliorating effect of NAC against alcohol induced elevation in LPO and reduction in SOD in the rat liver tissue.

The non-enzymic antioxidants reduced glutathione (GSH), vitamin-C and vitamin-E constitute the second line of defense which scavenges the free radicals that escape from decomposition by the enzymic antioxidants. Vitamin-E and vitamin-C are said to act synergistically to scavenge free radicals from the biological system. Thus, both vitamin-C and vitamin-E have been documented to protect the biological system against oxidative damage induced by xenobiotics (Buettner, 1993). GSH is a tripeptide which acts synergistically with vitamin-E in inhibiting oxidative stress and acts against lipid peroxidation (Chaudiere, 1994). The decrease in the levels of these antioxidant vitamins observed in this study is due to the reduction in the GSH levels, which in turn attributed to its over utilization in scavenging free radicals released during the metabolism of DMN. Reduction in the GSH and vitamin-C levels on DMN treatment has been reported in various studies (George, 2003; Farombi et al., 2008), and our results confirmed these findings. NAC post-treatment effectively protected the liver against DMN induced depletion in the levels of the GSH and antioxidant vitamins by restoring their levels towards normalcy, which is due to its free radical scavenging and potent antioxidant activity. NAC has been reported to exert its antioxidant effect firstly by indirectly facilitating the GSH biosynthesis and hence increasing GSH supply and secondly by directly reacting with reactive oxygen species and thus overcoming the oxidative stress (Ocal et al., 2004). Disturbance in the oxidant–antioxidant homeostasis caused by DMN treatment is further confirmed by a significant reduction in the activities of the glutathione metabolizing enzymes glutathione reductase (GR) and glutathione-*S*- transferase (GST). The fall in GR associated with GSH in DMN treated rats indicates that GSH-redox cycle is operating in an accelerated phase to maintain the GSH/GSSG homeostasis by replenishing the loss of GSH and by eliminating the toxic metabolites of DMN. Decrease in GST levels in DMN exposed rats could be due to the conjugation of GSH with the highly reactive electrophilic species liberated by this toxin, which could have caused a decrease in GST and GSH levels. Post-treatment with NAC significantly attenuated the fall in the status of GR and GST and protected the liver against DMN induced challenge of oxidative stress. As a GSH precursor NAC prevents the depression of GSH and its metabolizing enzymes and thus contributes indirectly to the attenuation of DMN induced oxidative stress which is clearly evidenced by a significant improvement in the GSH levels after NAC post-treatment. The protective effect of NAC is attributed to its ability to restore the total intracellular sulfhydryl pool, to maintain the intracellular GSH concentration, to assist in detoxification of toxic metabolites, and to its potential antioxidant action (Vendemiale et al., 2001).

The sodium/potassium (Na^+/K^+), magnesium (Mg^{2+}) and calcium (Ca^{2+}) ATPases function by transporting ions across cell membrane at the expense of ATP (Murray et al., 2003). From the studies conducted by Freel and Goldner (1981), it was proposed that the hepatic injury consequent to peroxidative damage to the lipid membrane results in alteration of structural and functional characteristics of parenchymal cell membranes and thus affects the activities of the membrane bound ATPases. These enzymes are sensitive to oxidative stress, formation of free radicals and hydroperoxides that are generated during DMN metabolism which might have made them vulnerable for their inhibition. A decline in the status of these membrane bound ATPases, is indicative of oxidative stress and derangement of hepatocellular membrane integrity on DMN administration. The restoration in the activities of the above membrane bound ATPases on NAC post-treatment could be due to prevention of the peroxidative damage to the membrane lipids and to its potential antioxidant and membrane stabilizing properties. Pascale et al. (1989) have shown that NAC prevents the inhibition of rat liver plasma membrane Na^+/K^+ ATPase levels on ethanol administration, by maintaining the intracellular GSH pool which might be the reason for its significant protection against DMN induced fall in the status of this enzyme.

The biochemical findings are supported by the histopathological observations, which reveal induction of hepatocellular damage and pre-malignant changes on DMN administration. DMN + NAC treated rats show improvement in the liver architecture indicating the ameliorative effect of NAC against DMN induced hepatotoxicity. NAC alone treatment did not produce any change in the histopathology of the liver tissue.

The results of the present study demonstrate that NAC could have effectively prevented the DMN induced oxidative stress and hepatocellular damage by two mechanisms: i) by directly scavenging the free radicals and ii) by indirectly facilitating GSH biosynthesis. Thus, NAC exhibits hepatoprotective, antioxidant and membrane stabilizing action, which is confirmed by the reversal in the status of the marker enzymes, reduction in lipid peroxidation, restoration of enzymic and non-enzymic antioxidants and membrane bound ATPases. These antioxidants protect cells against oxidative damage through the Nrf2-Keap-1-ARE signaling pathway. Nuclear factor erythroid 2-related factor 2 (Nrf2), a key transcription factor found in the cytosol as an inactive Nrf2-Keap-1 complex, upon signaling from reactive oxygen species insults dissociates and translocates into the nucleus and binds to the antioxidant response element (ARE) located in the promoter region of the genes encoding various antioxidants and phase II detoxifying enzymes. Thus Nrf2 regulates the gene expression of heme oxygenase-1, NAD(P)H dehydrogenase, quinone-1, CAT, SOD-1, GST, GPx, etc. (Kong et al., 2001). Curcumin has been shown to attenuate DMN induced oxidative stress via Nrf2 mediated induction of heme oxygenase-1 (Farombi et al., 2008). The thiol hypothesis reported by Li et al. (2004) reveals that NAC interferes with Nrf2 mediated activation of ARE by its reactive oxygen species scavenging action, by being a GSH precursor, and using its Src homology group to participate in electrophilic interactions allowing its direct binding to the toxicants. This hypothesis may be postulated as the mechanism by which NAC might exhibit its antioxidative effect against DMN induced oxidative stress.

In conclusion, our present study shows that NAC attenuates DMN induced oxidative stress and hepatocellular damage by virtue of its free radical scavenging, glutathione replenishing and potential antioxidant properties.

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