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Influence of silibinin on *N*-nitrosodimethylamine-induced changes in the genomic DNA and protein expression in the liver and lung tissue of rats

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Abstract

Background and Objectives: Though, several principles have been shown to protect the liver and lung against toxicity induced by the potent hepatotoxin i.e., *N*-nitrosodimethylamine (DMN), data regarding the protective ability of silibinin (SBN), a known hepatoprotective principle, extracted from the medicinal plant *Silybum marianum* is scanty. The objective of the present study is to evaluate the influence of SBN post-treatment against repeated DMN-induced changes in DNA, RNA and protein expressions in liver and lung tissue of rats.

Methods: Rats of either sex (n=6) were treated DMN (10mg/kg b.w.; i.p.) daily for first three days of every week for a period of three weeks (group II). Group III rats were treated on par with group II and from day 22 till day 35, they were post-treated SBN daily at a dose of 100mg/kg b.w., orally. Rats treated saline throughout the study period, served as control (group I). The status of nucleic acids (DNA, RNA) and expression of genomic DNA and protein were evaluated in liver and lung tissues.

Results: DMN alone treatment caused an increase in DNA and RNA accompanied by a fall in the expression of hepatocellular genomic DNA. This treatment also caused the over expression of a low molecular weight protein in the liver tissue of rats. Almost all the above abnormalities induced by repeated DMN treatments were significantly protected and reversed back towards normalcy in rats post-treated SBN. In the lung tissue, DMN alone treatment, caused a decrease in DNA, protein and an increase in RNA. This adversity was protected and reversed back towards normalcy in rats post-treated SBN. DMN alone treatment did not cause change in the expression of protein in lung tissue.

Conclusion: Noticeable changes in genomic DNA, protein expression, total protein and nucleic acids induced in the liver and lung tissue of rats treated repeated dose of DMN was significantly protected and reversed back towards normalcy on post-treatment with SBN. This protective effect is attributed to antioxidant and free radical scavenging properties of SBN. More studies are warranted to understand the precise mechanism of SBN protection against repeated administration of DMN-induced liver and lung damage.

Key words: *N*-nitrosodimethylsamine (DMN), protein expression, silibinin (SBN),

Introduction

Presences of *N*-nitroso compounds in foods have been implicated as the cause for hepatotoxicity, especially carcinogenicity of the liver. *N*-nitrosodimethylamine (DMN), a well-studied group of nitrosamine family is a powerful animal carcinogen, capable of inducing benign and malignant tumors in variety of tissues including the liver, kidney, lung and nasal cavity (1,2). Several investigators have furnished large

volumes of data conforming that substantial human exposure to DMN occurs via the diet, tobacco smoke and certain occupational settings as well as through its endogenous formation in the human body (1-3). Presences of DMN in Kashmiri foods have been related with high incidence of oesophageal cancer, which is prevalent in this region (4). Exposure of rats and mice to low dose of DMN have been shown to increase the incidence of tumors, various incidences of adenomas, carcinomas and sarcomas in the lung, liver, kidney and heart (2). Repeated administration of DMN was shown to develop hepatic fibrosis, alterations in protein metabolism and changes in marker enzymes of hepatotoxicity in serum and liver tissue of rats (5).

Hepatotoxicity and hepatic fibrosis induced by short-term and long-term administration of DMN has been shown to be alleviated and reversed back towards normalcy by administration of inhibitors of monoamine oxidases (6) and extracts derived from various medicinal plants (7-9). However, data on the protective ability of silibinin (SBN), a known hepatoprotective agent extracted from the plant *Silybum marianum* against DMN-induced hepatotoxicity and changes in genomic DNA, RNA and protein are scanty. The aim of this study is to evaluate the influence of SBN post-treatment on DMN-induced changes in genomic DNA, RNA and protein expressions in the liver and lung tissue of rats.

Materials and Methods

N-nitrosodimethylamine (DMN), silibinin (SBN), agarose, acrylamide and *N,N'* methylene bisacrylamide were purchased from Sigma-Aldrich Chemicals, USA. DNA marker Hind III λ (23,000 to 500 bp) and protein molecular weight markers (116.0 to 14.4 kda) were purchased from M/s Medox Biotech, Chennai, India. Tris-saturated phenol:choloroform:isoamyl alcohol was purchased from SRL, Mumbai, India. All the other chemicals used in this study were procured locally and they were of analytical grade.

Animals

Wistar albino male rats weighing between 150 to 180 gms, obtained from the central animal house facility of Dr. ALM Post Graduate Institute of Medical Sciences, University of Madras, Taramani campus, Chennai-113, were used in this study. They are housed in polypropylene cages over husk bedding in controlled environmental conditions (temperature 23 ± 4°C; relative humidity 50-70%; 12 h dark/light cycle) and were provided food and water *ad libitum*. Animal experiments were performed after getting prior approval from Institutional Animal Ethics Committee of University of Madras, governed by guidelines prescribed by Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of. India.

Treatment Schedule

Rats were divided into three groups at random and each group comprised six animals. Group I rats served as control and were given physiological saline throughout the study period. Rats belonging to group II and group III were treated DMN (10mg/kg, i.p.) during the first three days of each week, for three weeks as described by George and Chandrakasan (5). From the beginning of 4th week i.e., from day 22 until day 35 (for two weeks), while the group II rats were left with out any treatment, that of group III were treated SBN orally, daily at a dose of 100mg/kg b.w. All the rats were sacrificed on day 36 under mild ether anesthesia followed by cervical decapitation. The rats were dissected and the livers and lungs were harvested quickly. They were washed in physiological saline to remove blood clots and other unwanted tissue materials. All the tissues were stored at -20°C until further analysis. Around 30 to 40mg of liver and lung tissues were homogenized in tris-HCl buffer (0.1M; pH-7.4) and the homogenates were centrifuged at 10,000 rpm at cold (4°C). The clear supernatants were used for protein analysis and electrophoresis.

Biochemical assay and electrophoresis

The liver and lung tissues were homogenized in 20%

TCA and subsequently treated with 95% ethanol to remove protein debris and lipids. The lipid free residue was re-suspended in water and TCA and was heated for 15 min at 90°C to dissolve the pellet containing nucleic acid and to subsequently split both DNA and RNA. This was further purified by centrifugation and the DNA content was estimated calorimetrically using diphenylamine reagent and RNA by orcinol reagent as described by Schneider (10). The high molecular weight DNA from tissue sample was isolated into aqueous phase in the presence of phenol:chloroform:isoamyl alcohol and was subsequently precipitated by absolute ethanol as described by Garner (11) . The DNA extracted from tissues was subjected to gel electrophoresis technique in 1% agarose gel as described by Williams and Rapley (12). The gel documentation images were photographed using software (Alpha Innotech Image Analyzer, Alpha EaseTM FC stand alone software version-6.6.6.14 for windows XP).

The liver and lung homogenates were placed in equal volume of sample treatment buffer and heated at 100°C for one min to enable denaturation of proteins. Around 5 to 10 µl of this denatured protein sample was applied on the acrylamide gel and electrophoresis was carried out as detailed by Weber and Osborn (13). The gels were stained with coomassie brilliant blue and de-stained in acetic acid with ethanol. The distained gels were photographed in gel documentation system (Alpha Innotech, USA) for comparison of intensity of bands between a standard molecular weight marker protein and the proteins separated from the tissue samples.

The total protein in liver and lung tissue homogenates were estimated as described by Lowry et al (14).

Statistical analysis

The data are subjected to one-way analysis of variance (ANOVA). Post-hoc multiple comparison was done by Tukey's test to evaluate the significance difference of means between various treatment groups. The values are presented is mean ± S.D. and $p < 0.05$ was considered significant. The above statistical programs were performed using SPSS statistical software package (version-7.5).

Results

Repeated DMN administration (Group II) caused nearly five-fold increase in DNA content of the liver and this adversity was further enhanced in rats post-treated SBN with DMN (Group III), as compare to the saline treated control (Table 1). In the lung tissue, DMN alone treatment caused a highly significant decrease in DNA and this adversity was protected and reversed back towards normalcy in rats post-treated SBN (Table 2). The status of both liver and lung tissue RNA were elevated highly significantly in rats treated DMN alone and this adversity was significantly protected by SBN post-treatment (Table 1, 2). SBN also protected against the fall in the liver protein induced by DMN alone treatment and reversed it towards normalcy (Table 1). The lung proteins, however, remain unaltered in both DMN alone and DMN + SBN treatments and they are comparable to those of control (Table 2).

The genomic DNA expression profiles of liver and lung tissues of rats treated DMN alone and DMN + SBN are presented in Fig. 1 and 2. It is interesting to observe that rats treated DMN alone show a fall in the expression of genomic DNA (at 9416 bp) in both the liver and lung as compared to saline treated control. This abnormality was found to be reversed back towards normalcy in rats post-treated with SBN, only in the liver and not in the lung.

The SDS-PAGE electrophoresis of liver and lung tissue homogenates of rats are presented in Fig. 3 and 4. In DMN alone treated rats, there was a high expression of low molecular weight proteins, little below the Rf of standard protein i.e., 14.4 kda, as compare to the saline treated control. The enhanced expression of this low molecular weight protein observed in DMN treated rats were completely prevented, and reversed back towards normalcy and were comparable to the saline treated controls in rats post-treated SBN (Fig. 3). Almost identical pattern of electrophoretic mobility of different protein fractions were observed in the lung tissue of rats treated DMN alone as well as DMN + SBN, and they were comparable to those of control group (Fig. 4).

Discussion

It is a well-established fact that DMN is a potent hepatotoxin, carcinogen and mutagen in experimental animals (2,15). While exposure to low dose of DMN is shown to cause chronic liver injury with varying degrees of necrosis, fibrosis and nodular degeneration in both liver and lung (1), its repeated administration was shown to induce apoptotic like changes, dysplasia, severe necrosis, bridging fibrosis and collapse of parenchymal framework of liver (16). In the present study, we observed a five-fold increase in the DNA and highly significant increase in RNA (Table 1) in the liver tissue of rats treated with repeated doses of DMN. The disruption in nucleic acid homeostasis is also evidenced by five-fold increase in RNA and a significant decrease in DNA in the lung tissue of rats treated DMN alone (Table 2). DMN-induced damages in genomic DNA are further evidenced by a noticeable fall in the expression of both liver and lung tissue DNA (Fig. 1 and 2). These observations clearly demonstrate the genomic toxicity of repeated DMN exposure. Several studies have suggested that DMN administration lead to the methylation of DNA and it could occur at more than a dozen different positions, leading to formation of N^7 guanine (17,18). George et al (16) reported a progressive increase in DNA in the liver tissue of rats repeatedly treated DMN, three days per *week for three weeks. Increase in DNA synthesis* and cellular proliferation of hepatic stellate cell DNA was also reported (19) and our results are in agreement with these reports. In the present study, the cause for fall in the DNA in lung tissue is not known. However, this effect could be attributed to the differential bioavailability of DMN in liver and lung tissues and different mechanisms of DNA adduct formation in these tissues. DMN alone treatment caused a fall in the expression of genomic DNA in both liver and lung at high molecular weight base pairs (Fig. 1 and 2) and this adversity has not been reported in the previous literature. The reduction in the expression of DNA could be attributed to the increased adduct formation of DMN metabolites with the hepatocellular genomic DNA.

The concentrations of RNA, especially the mRNAs for type I, II and IV collagens were reported to be the early events in the development of DMN-induced hepatic fibrosis. The expressions of mRNAs for the above collagens were reported to show a definite increase a week after DMN treatment and the largest increase is reported 21 days after its repeated administration (20). Several animal models have also shown an increase in mRNA expression in DMN-induced hepatic fibrosis (21,22). In the light of these reports, the increase observed in the RNA levels in both liver and lung tissues of rat treated repeated doses of DMN alone could be attributed to the onset of fibrosis, consequent to the increased collagen deposition in the above tissues.

In the present study, a highly significant fall in status of both liver and lung proteins (Table 1 and 2) is observed in rats treated DMN alone. Significant decrease in total liver proteins is reported in rats treated DMN (5,20) and our present observations are in agreement with these reports. An increase in protein breakdown and its turnover has been reported in patients with cirrhosis (23) and this adversity was attributed to the increased in catabolism of protein consequent to extreme centrilobular necrosis, cirrhosis and other liver tissue damage induced during repeated DMN administration (5).

In the present study, the protein electrophoresis of liver tissue shows an increase in the expression of *a low molecular weight protein (i.e., at Rf below* 14.4 kda). This is a novel observation not reported in previous literatures. The significance of this low molecular weight protein function is unknown. However, the increase in the expression of this protein may serve as a molecular biomarker in the detection and identification of DMN-induced hepatotoxicity manifested as hepatic necrosis, fibrosis and DNA damage. Nevertheless, further studies are warranted to evaluate the significance of this low molecular weight protein.

Metabolic conversion of DMN in the liver is reported to be the cause for all the DMN-induced hepatotoxicity in experimental animals (1,24). It is hypothesized that the metabolism of DMN involves α-hydroxylation, which is mediated by the cytochrome P450 mixed function oxidases. It is believed that this

Table 1:

Effect of DMN + SBN treatments on DNA, RNA and protein in liver tissue of rats

Treatment groups (n=6)	Parameters DNA (µg/gm tissue)	RNA (µg/gm tissue)	Protein (mg/gm tissue)
Control (group-I)	44.74 ± 3.72	11.68 ± 1.15	120.31 ± 15.89
DMN alone (group-II)	220.83 ± 10.67 a***	17.14 ± 1.37 a***	89.62 ± 13.48 a**
DMN+ SBN (group-III)	258.11 ± 9.58 a,b***	8.83 ± 1.51 a**,b***	100.25 ± 12.68 a,b**

Values presented are mean ± S.D. Multiple comparisons were performed by Tukey's test.
a - Group-I compared to Groups II and III; b - Group-II compared to Group III.
ns - not significant; ** $p < 0.01$; *** $p < 0.001$.

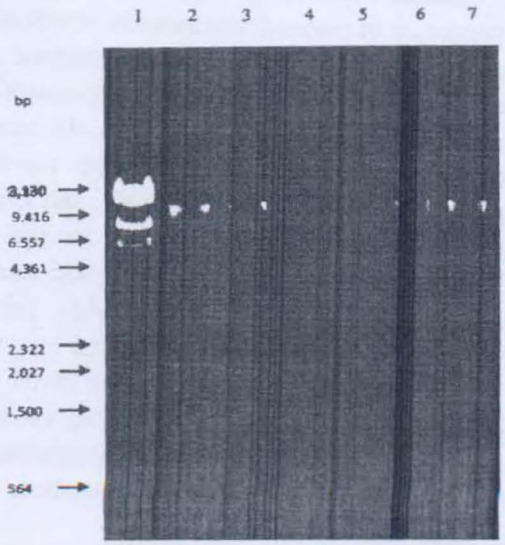
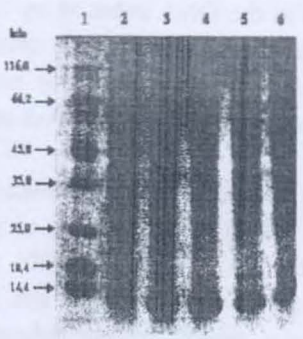


Table 2:

Effect of DMN + SBN treatments on DNA, RNA and Protein in lung tissue of rats

Treatment groups (n=6)	Parameters DNA (µg/gm tissue)	RNA (µg/gm tissue)	Protein (mg/gm tissue)
Control (group-I)	230.92 ± 17.37	2.10 ± 1.81	59.68 ± 7.80
DMN alone (group-II)	167.32 ± 30.91 a***	11.44 ± 1.77 a***	69.12 ± 11.17 a**
D M N + S B N (group-III)	209.43 ± 8.17 a**,b***	4.64 ± 0.77 a*,b***	74.72 ± 14.40 a,b**

Values presented are mean ± S.D. Multiple comparisons were performed by Tukey's test.
a - Group-I compared to Groups II and III; b - Group-II compared to Group III.
ns - not significant; ** $p < 0.01$; *** $p < 0.001$.



α-hydroxylated amine is decomposed enzymatically to an unstable monomethyl nitrosamine, resulting in the formation of carbonyl compounds, which is said to undergo prototropic shift to form diazotic acid, methyl diazohydroxide and other intermediates such as methyl diazonium ions. All these electrophilic compounds are believed to bind with the tissue macromolecules such as DNA, RNA and protein, resulting in hepatotoxicity (25,26). In the light of these reports, it could be suggested that the alterations noticed in the nucleic acids, genomic DNA and proteins, in both the liver and lung tissue of rats treated with repeated doses of DMN could have been caused consequence to the release of its highly reactive electrophilic metabolites of DMN and their adduct formation with these tissue macromolecules.

In the present study, post-treatment of SBN in DMN pre-treated rats did not prevent the five-fold increase in DNA in the liver tissue. On the contrary, this treatment caused a further increase in DNA as compare to DMN alone treated rats (Table 1). This observation indicates the fact that SBN treatment further enhances the net DNA synthesis in the liver tissue as a healing mechanism to compensate for the loss in the DNA induced by DMN toxicity. SBN post-treatment significantly protected against increase in RNA and decrease in protein induced by DMN treatment in both the liver and lung tissue (Table 1,2).

The above treatment also significantly protected against the fall in the expression of genomic DNA in the liver tissue. Further, SBN post-treatment also reversed the increase in the expression of low molecular weight protein induced by repeated DMN administration. The protective ability of SBN post-treatment against DMN-induced alterations in nucleic acids, genomic profiles and protein expressions has not been reported in the previous literature.

SBN, a major constituent of the flavonolignan, i.e., silymarin, extracted from the medicinal plant *Silybum marianum* has been shown to be good hepatoprotective agents in experimental animals and humans. It is reported to offer protection against various drugs and chemicals induced liver damage such as cirrhosis, hepatitis, jaundice and alcoholic liver diseases. The hepatoprotective effects of SBN is attributed to direct or indirect antioxidant, membrane stabilizing and free radical scavenging activity (27,28). Moreover, SBN has been shown to inhibit cytochrome P450 metabolizing enzyme and thus protects the liver against many drugs and alcohol-induced liver toxicity (29,30).

In light of these reports, it could be suggested that the protective effect of SBN against changes in nucleic acids, genomic profile and protein expression in liver and lung tissues induced by DMN could be attributed to the antioxidant and free radical scavenging activities. The precise mechanism of protective effect of SBN against DMN-induced toxicities in the liver and lung tissues are not clearly established and further studies are warranted on these lines.

Conclusion

In conclusion, noticeable changes observed in genomic profiles of DNA, protein expression, total protein and nucleic acids in both the liver and lung tissue of rats induced by repeated administration of DMN could be significantly protected and reversed back towards normalcy by post-treatment with SBN. This beneficial effect could be attributed to the hepatoprotective, antioxidant and free radical scavenging properties of SBN.

Further studies are warranted to understand the precise mechanism of SBN protection against repeated DMN-induced liver and lung damage.

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