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Full Length Research Paper

Attenuation of N-nitrosodiethylamine-induced liver carcinogenesis in rats by naturally occurring diallyl sulfide

Sherine M. Rizk and Safinaz S. Ibrahim*

Biochemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt.

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The present study was aimed to investigate the chemopreventive effects of diallyl sulfide (DAS), organosulfur compounds present in high amounts in garlic, against N-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis in rats. NDEA treatment to rats resulted in significantly elevated levels of serum aspartate transaminase, alkaline phosphatase, lactate dehydrogenase and sorbitol dehydrogenase along with significant decrease in serum total protein and albumin/globulin ratio, which are indicative of hepatocellular damage. Hepatic malondialdehyde and nitric oxide levels were also elevated. Hepatic glutathione, protein thiol, glutathione reductase, glutathione peroxidase, glutathione-S-transferase, gammaglutamyl cysteine synthetase and gammaglutamyl transferase were significantly increased in NDEA-treated group as compared to the control rats indicating disturbances in oxidant/antioxidant status. A significant decrease in hepatic ATP level was recorded indicating failure of energy metabolism. Administration of DAS to the NDEA- treated rats resulted in restoration of most of enzymatic and non enzymatic liver function tests. Also, liver content of most of the measured oxidants and antioxidant systems, enzymatic as well as non enzymatic, were normalized. Moreover, administration of DAS to NDEA-treated rats showed significant increase in pyruvate, ATP contents and lactate dehydrogenase activity, along with decrease in lactate and lactate/pyruvate ratio as compared to NDEA-treated rats. The histopathological examination of the liver sections confirmed these results. In conclusion, DAS could attenuate NDEA- induced hepatocarcinogenesis by improving the oxidant/antioxidant balance as well as the energy status of the hepatic tissue.

Key words: N-nitrosodiethylamine, hepatocarcinogenesis, diallyl sulfide, energy metabolism, oxidative stress, antioxidant systems.

INTRODUCTION

Allium vegetables, including garlic are used throughout the world for their sensory characteristics as well as for their apparent health benefits. To date, around more than 10,000 publications from all the world have confirmed the beneficial effects of garlic and its compounds including the reduction of risk factors for cardiovascular diseases and cancer, stimulation of the immune function, enhanced foreign compound detoxification and radio protection (Manivasagam et al., 2005). Epidemiologic studies have reported that high consumption of garlic reduces the risk of gastric and colon cancer (Fleischauer and Arab, 2001). High quantities of garlic are required to produce a beneficial medicinal effect. However, high consumption of crushed raw garlic was reported to produce many undesirable clinical effects such as anemia, weight loss, growth reduction and decrease of caecal microflora and serum protein (Moihara et al., 2006). Therefore, the use of garlic as crushed raw material is not preferred. Experimental investigations have provided evidence that organosulfur compounds present in high amounts in garlic, one of these namely diallyl sulfide (DAS), account for its anticarcinogenic activity (Milner, 2006). The mechanisms responsible for these chemopreventive effects have not been fully elucidated. Hepatocellular cancer formation is a multi-factorial process and

*Corresponding author. E-mail: safinazsibrahim@cu.edu.eg. Mobile: 002 010 1595025; Fax: 002-02-23628426.

possible mechanisms leading to cancer are not very clear. N-nitrosodiethylamine, is a potent hepatocarcino genic dialkyl nitrosamine present in tobacco smoke, water, and cheese, cured and fried meats and in a number of beverages (Rajesh and Kuttan, 2000). Increasing evidences point to reactive oxygen species (ROS) induced damage (Czeczot et al., 2006) along with disturbances in energy metabolism (Nishikawa et al., 1996) as important contributors to the liver disease. Thus, the aim of the present study was to evaluate the chemopreventive efficiency of DAS against NDEA-induced hepato-carcinogenesis in rats. Hepato-carcinogenesis was induced by oral administration of NDEA. The protective effect of DAS on NDEA-induced liver carcinoma in rats was assessed by evaluating the oxidative stress biomarkers, enzymatic and non-enzymatic antioxidants and the energy metabolism status in liver tissues, along with liver function tests.

MATERIALS AND METHODS

Chemicals and drugs

N-nitrosodiethylamine (NDEA); diallyl sulfide (DAS); enzymes and coenzymes were obtained from Sigma (St. Louis, MD, USA). Other chemicals were from Analar grade or from purest grade available.

Animals used

Male albino rats of Wistar strain (weighing 170 - 200 g), bred in the central animal house of Faculty of Pharmacy, Cairo University (Cairo, Egypt) were used in the study. The animals were housed in groups (10 each) and kept at constant environmental and nutritional conditions throughout the experimental period. The experimental protocols were approved by the Ethical Committee of Cairo University, Cairo, Egypt.

Experimental groups and protocol

Animals were divided into the following groups: Group (1): NDEA-treated group in which hepatocarcinogenesis was induced by a technique modified from Karimov et al. (2003). NDEA was dissolved in saline (8 mg/ml saline) and given orally to rats in a dose of 20 mg/kg body wt/day for 5 days a week for 9 weeks, followed by 10 mg/kg body wt/day for 5 days a week for another 6 weeks. Group (2): animals received NDEA as in group 1 and in addition, they were orally administered DAS. DAS was dissolved in corn oil (80 mg/ml corn oil) and administered orally at a dose of 200 mg/kg body wt/day (Sheen et al., 1999) for 5 days a week for the total period of experi-ment. Group (3): the control groups, which normal rats were received saline or corn oil separately at a dose similar to that in group (1) and (2), for the entire of the experimental period. Treated and control animals were allowed free access to water and standard chow diet up to the end of experimental period.

Biochemical analysis

Blood sampling: At the end of the experimental period, all the animals were sacrificed. Blood sample was collected in heparinized tubes and centrifuged at 600 x g for 15 min. Plasma was used for the estimation of total protein (Weichselbaum, 1946) and albumin (Doumas et al., 1971). Serum was prepared separately and was used for the determination of aspartate transaminase (AST) according to the method of Reitman and Frankel (1957) using a kit provided by Bicon (Germany). Alkaline phos-phatase (ALP) was estimated according to the modified method of Belfield and Goldberg (1971), using kit pro-vided by BioLab (France). Lactate dehydrogenase (LDH) (Buhl and Jackson, 1978) and sorbitol dehydrogenase (SD) (Leissing and McGuinness, 1982) were also mea-sured.

Tissue sampling: The liver was removed, rinsed with ice-cold saline and blotted dry. Weighed liver tissues were treated differently for various estimation as follows:

i.) Two portions were used to prepare 10% homogenate in 1.15% KCl and 5% homogenate in 3% sulfosalicylic acid, centrifuged at 1000 x g at 4^{o}C for 20 min and the supernatants were used for the assay of malondialde-hyde (MDA) (Yoshioka et al., 1979) and glutathione (GSH) (Srivastava and Beutler, 1968) respectively.
ii.) Another portion of liver tissue was homogenized in Tris-sucrose buffer, pH 7.4 to obtain a 5% homogenate, using Potter-Elvejhem glass homogenizer and centri-fuged at 2000 x g, at 4^{o}C for 10 min. The supernatant was used for estimation of nitric oxide (NO) (Harold et al., 1992) and protein thiol (PSH) (Koster et al., 1986).
iii.) Portion of the liver was homogenized in 5% metaphosphoric acid, centrifuged at 3000 x g at 4ºC for 15 min, and the supernatant was used for the estimation of pyruvate and lactate according to the methods of Mohun and Cook (1957) and David (1999) respectively.
iv.) An additional portion of the liver tissue was homogenized in 3 ml of ice-cold 3 M percholoric acid, using a Potter-Elvejhem glass homogenizer. EDTA (12.5 ml, 1 mM) was added and the mixture was centrifuged at 1000 xg, at 4^{o}C for 1 hr. The supernatant was used treated for the estimation of ATP (Lowry et al., 1964).
v.) A portion of the liver tissue was homogenized in Tris -sucrose buffer pH 7.4 to prepare a 10% homogenate, centrifuge at 105,000 x g, at 4^{o}C for 30 min, using a Dupont-Sorvall Ultracentrifuge (USA), to obtain the cyto-solic fraction, which was used for the assay of glutathione peroxidase (GPX) (Arthur and Boyne, 1985), glutathione reductase (GR) (Long and Carson, 1961), glutathione-S- transferase (GST) (Habig et al., 1974), gamma glutamyl cysteine synthetase (GCS) (Sekura and Meister, 1977), gamma glutamyl transferase (GT) (Szasz, 1969), LDH (Buhl and Jackson, 1978) and glucose-6-phosphate dehydrogenase (G6PD) (Sie et al., 1967).

Protein concentrations of the above supernatants wereestimated by the method of Lowry et al. (1951). Portions of liver tissue from different groups were stored in formol-saline (10%) for histopathological analysis. Liver sections were stained using eosin yellow-hematoxin, followed by examination using light microscopy.

Statistical analysis: Results are given as the Mean ± SEM. Comparison of the experimental groups with con-trol was carried out by one way Analysis Of Variance (ANOVA) followed by Kruskal-Wallis test (Armitage and Berry, 1987). $P < 0.05$ was considered significant.

RESULTS

NDEA treatment caused significant decrease in plasma total protein as well as A/G ratio. DAS administration to NDEA-treated rats effectively normalized these previous parameters. Also, NDEA-treated rats showed significant increase in serum AST, LDH and SD activities, along with significant decrease in ALP activity com-pared to control. DAS-administration produced normali-zation in serum AST, ALP, LDH and SD activities compared to

Table 1. Serum parameters of normal, NDEA- and DAS-treated NDEA-rats.

Control Parameters	Normal control	NDEA rats	DAS-treated NDEA-rats
AST (IU/L)	22.3 ± 0.785	32.6 ± 1.15 [a]	22.9 ± 0.75 [b]
ALP (IU/L)	74.4± 4.36	230.2 ± 6.94 [a]	115.6± 10.62 [ab]
LDH (IU/L)	451 ± 19.7	725.12 ± 25.7 [a]	436.32 ± 30.4 [b]
SD (IU/L)	33.9 ± 2.45	53.7 ± 2.99 [a]	34.5 ± 0.92 [b]
Total Protein (g/dl)	8.09 ± 0.20	6.47 ± 0.31 [a]	7.86 ± 0.154 [b]
A/G ratio	1.39 ± 0.06	0.87 ± 0.06 [a]	1.3 ± 0.081 [b]

Results expressed as Means ± S.E.M.
[a] Significantly different from normal control at $p < 0.05$.
[b] Significantly different from NDEA-treated rats at $p < 0.05$.

Table 2. Liver oxidative stress indices in normal, NDEA- and DAS-treated NDEA-rats.

Control Parameters	Normal control	NDEA-rats	DAS-treated NDEA-rats
MDA (nmoles/ g liver)	55.6 ± 3.41	90.4 ± 8.01 [a]	66.8 ± 3.30 [a]
NO (nmoles/ g liver)	139 ± 9.7	344 ± 14.9 [a]	187 ± 9.2 [ab]

Results expressed as Means ± S.E.M.
[a] Significantly different from normal control at $p < 0.05$.
[b] Significantly different from NDEA-treated rats at $p < 0.05$

Table 3. Liver non-enzymatic antioxidant status in normal, NDEA- and DAS-treated NDEA-rats.

Control Parameters	Normal control	NDEA-rats	DAS-treated NDEA-rats
GSH (mg/g liver)	1.5 ± 0.104	3.82 ± 0.149 [a]	2.57 ± 0.17 [ab]
PSH (nmole/mg protein)	152.5 ± 7.03	224.25 ± 15.4 [a]	168.7 ± 9.13 [b]

Results expressed as Means ± S.E.M.
[a] Significantly different from normal control at $p < 0.05$.
[b] Significantly different from NDEA- treated rats at $p < 0.05$.

Table 4. Liver enzymatic antioxidant status in normal, NDEA- and DAS-treated NDEA-rats.

Control Parameters	Normal control	NDEA-treated rats	DAS- treated NDEA-rats
GR (nmol /mg protein/min)	80.1 ± 2.53	101 ± 5.95 [a]	78.5 ± 2.30 [b]
GPX (nmol/mg protein/min)	324.36 ± 7.6	397.2 ± 13.16 [a]	246.6 ± 8.04 [ab]
GST (nmol/mg protein/min)	246.3 ± 12.4	391.8 ± 9.68 [a]	297.5 ± 17.3 [b]
GCS (nmol/ mg protein/min)	6.01 ± 0.445	8.37± 0.244 [a]	6.9 ± 3.22 [b]
GT (U/mg protein)	2.12 ± 0.178	4.8 ± 0.304 [a]	1.75 ± 0.108 [b]
G6PD (mol/mg protein/min)	27.85 ± 2.29	47.4 ± 1.48 [a]	54.05 ± 4.04 [a]

Results expressed as Means ± S.E.M.
[a] Significantly different from normal control at $p < 0.05$.
[b] Significantly different from NDEA- treated rats at $p < 0.05$.

NDEA-treated rats (Table 1).

NDEA-treated rats showed significant increase in MDA and NO levels compared to control animals. Treatment with DAS produced normalization of MDA, along with significant decrease in NO content compared to NDEA-treated group value (Table 2).

NDEA adminstration produced significant increase in GSH and PSH contents compared to control. Treatment with DAS resulted in significant decrease in GSH as well as normalization in PSH compared to NDEA treated rats (Table 3).

Table 4 illustrated that NDEA administration to rats

Table 5. Liver energy status in normal, NDEA- and DAS-treated NDEA-rats.

Control Parameters	Normal control	NDEA-rats	DAS-treated NDEA-rats
Lactate (µmol/g liver)	2.27 ± 0.16	2.08 ± 0.094	1.41 ± 0.08 [ab]
Pyruvate (µmol/g liver)	0.11 ± 0.007	0.103 ± 0.008	0.15 ± 0.007 [ab]
Lactate/Pyruvate ratio	20.64 ± 1.65	17.37 ± 0.9	11.31 ± 0.76 [ab]
LDH (moles/mg protein/min)	0.90 ± 0.021	0.90 ± 0.043	1.05± 0.028 [ab]
ATP (mol/g liver)	5.69 ± 0.32	4.04 ± 0.12 [a]	4.91 ± 0.0856 [ab]

Results expressed as Means ± S. E.M.
[a]Significantly different from normal control at $p < 0.05$.
[b]Significantly different from NDEA- treated rats at $p < 0.05$

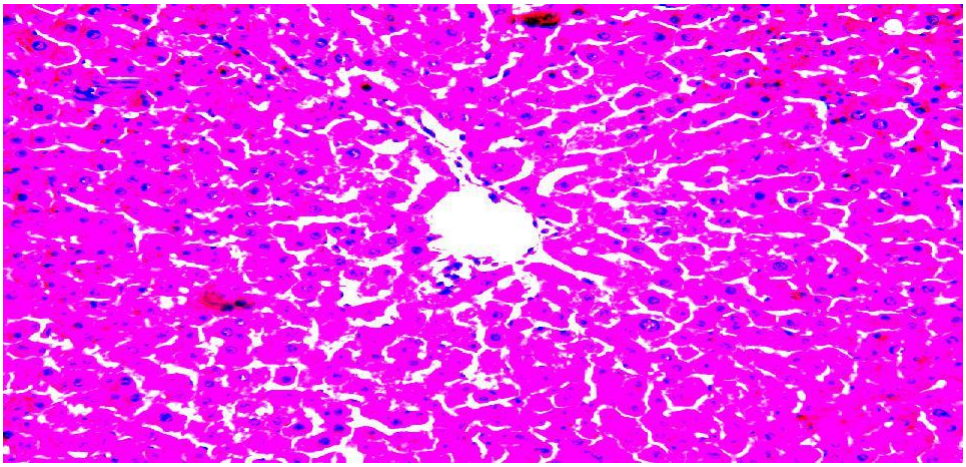

Figure 1A. Photomicrograph of the liver section from normal rat (X = 100).

produced significant increase in cytosolic GR, GPX, GST, GCS, GT and G6PD activities compared to control. Treatment with DAS resulted in restoration of GR, GST, GCS, and GT activities along with significant decrease in GPX activity as compared to normal well as NDEA-treated group. As shown in Table 5, NDEA-treated rats showed a significant decrease in ATP level as compared to control group. Treatment with DAS resulted in signifi-cant decrease in liver lactate and lactate/pyruvate ratio, significant elevation in pyruvate and LDH activity and ATP content compared to normal and NDEA-treated group.

Figure 2a Photomicrograph of the liver section from NDEA-treated rats (X = 100). The encircled area: multiple nucleoli; short arrows: pyknotic; arrow: intranuclear va-cuole; V: cytoplasmic vacuoles; Inf: cellular infiltration.

The histopathological examination of liver sections of NDEA- treated rats showed multiple nucleoli, pyknotic nuclei, intranuclear and cytoplasmic vacuole, cellular infil-tration (Figure 1B) and hyperchromatic malignant nuclei (Figure 1C). Liver tissues of the DAS-treated NDEA rats showed some degenerative changes and vacuolated cytoplasm (Figure 1D), while some sections showed more or less normal hepatic lobule architecture (Figure 1E) compared to the control (Figure 1A).

DISCUSSION

An understanding of how cancer may be prevented is one of the key objectives of the recent researches. This can be achieved to some extent by using chemopre-ventive agents, naturally occurring or synthetic, that can suppress or prevent the processes of tumor develop-ment. Therefore, it is essential to identify agents as well as to evaluate their efficacy and to elucidate their mecha-nisms of action.

In the present study, serum obtained from tumor bear-ing rats showed significant increase in AST, ALP, LDH and SD activities along with significant decrease in plasma total protein and A/G ratio compared to control animals. The elevation of these enzyme activities was indicative of the toxic effect of NDEA on the liver tissue. It is known that N-nitroso compounds act as strong carcino-gens in various mammals including primates (Swenberg et al., 1991). NDEA has been shown to be metabolized by cytochrome P-450 IIE1 (CYP 2E1) to its active ethyl radical metabolite, which could interact with DNA causing mutation and carcinogenesis (Anis et al., 2001). Admini-stration of DAS to NDEA treated rats showed restoration of AST, ALP, LDH, SD activities and plasma total protein and A/G ratio towards normal. Such reverse in serum enzyme activities could be attributed to the ability of DAS

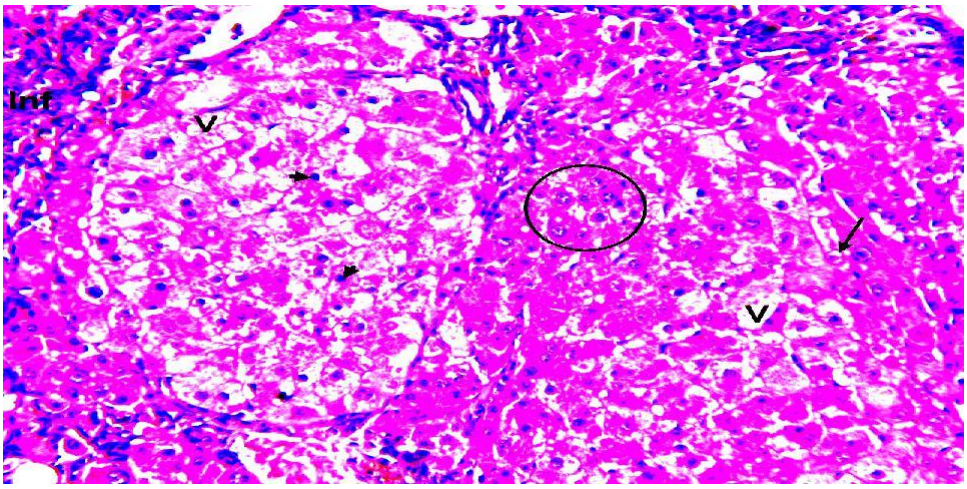

Figure 1B. Photomicrograph of the liver section from NDEA-treated rats (X = 100). Encircled area: multiple nucleoli; short arrows: pyknotic; arrow: intranuclear vacuole; V: cytoplasmic vacuoles; Inf: cellular infiltration.

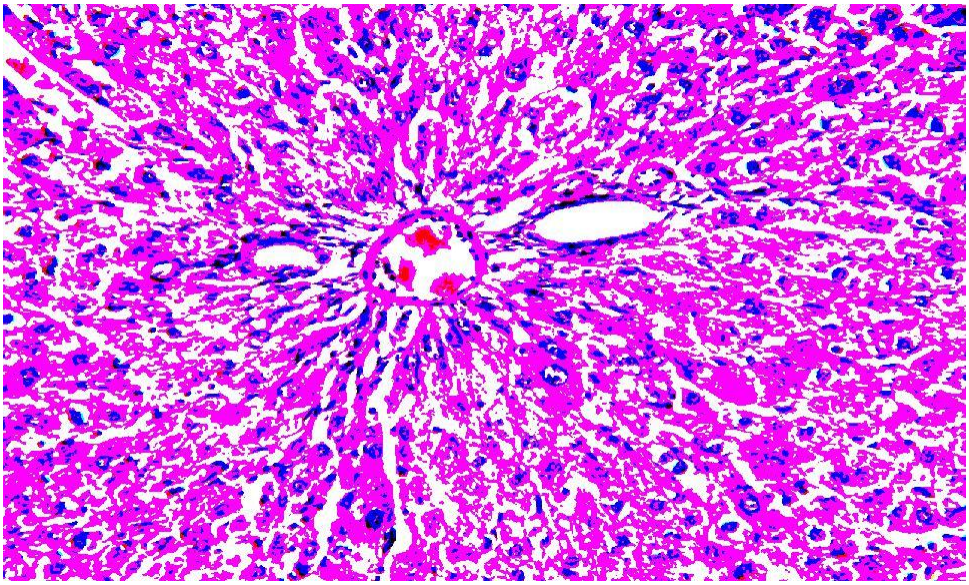

Figure 1C. Photomicrograph of the liver section from NDEA-treated rats (X = 100) showing hyperchromatic malignant nuclei.

to inhibit CYP 2E1 activity, presumably by serving as a competitive inhibitor, leading to a decrease in the formation and/or bioactivation of these nitrosamines (Yang et al., 2001).

Reactive oxygen species (ROS) are known to promote oxidative damage of the vital cellular constituents such as DNA, lipids and proteins and to cause alterations in the signal transduction pathways that control the expression of genes required to execute cell death (Klaunig and Kamendulis, 2004). In variety of tissues, generation of ROS leads to increased oxidative stress. In the present study, increased level of MDA as oxidative stress biomarker in the liver of tumor bearing animals could be ascribed to the excessive generation of free radicals.

ROS are produced during the metabolism of NDEA or during the process of carcinogenesis (Sundaresan and Subramanian, 2003) . MDA, owing to its high cytotoxicity and inhibitory action on protective enzymes is suggested to act as a tumor promoter and a co-carcinogen. Decreased levels of MDA in DAS-administered rats showed that DAS could scavenge free radicals in the rat liver tissue. DAS was found to have preventive properties against 4-hydroxynonenal, a key reactive aldehyde produced during lipid peroxidation (Fanelli et al., 1998).

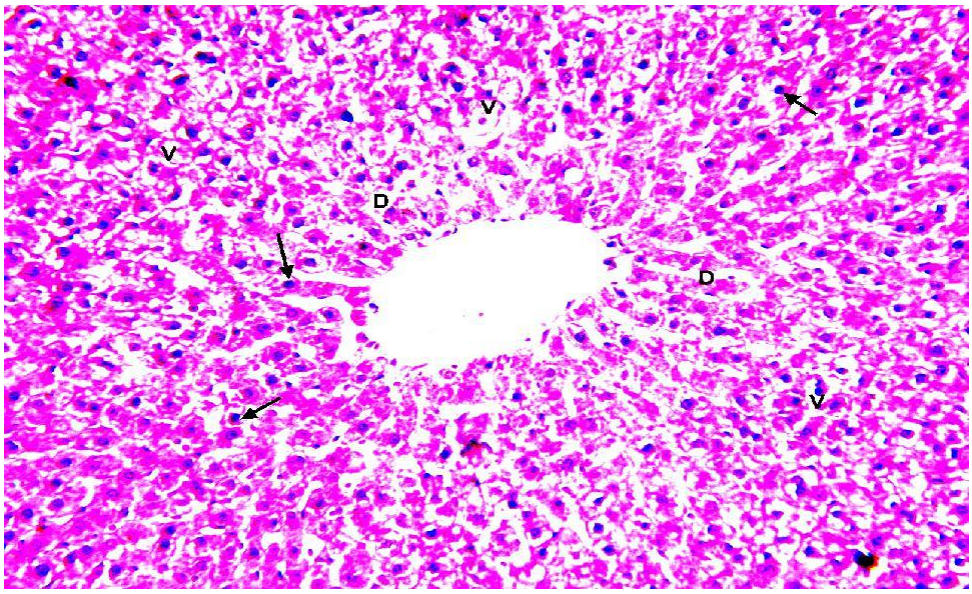

Figure 1D. Photomicrograph of the liver section from DAS-treated NDEA rats (X = 100). V: some degenerative changes; vacuolated cytoplasm; Arrows: pyknotic nuclei; D: dilated sinusoids.

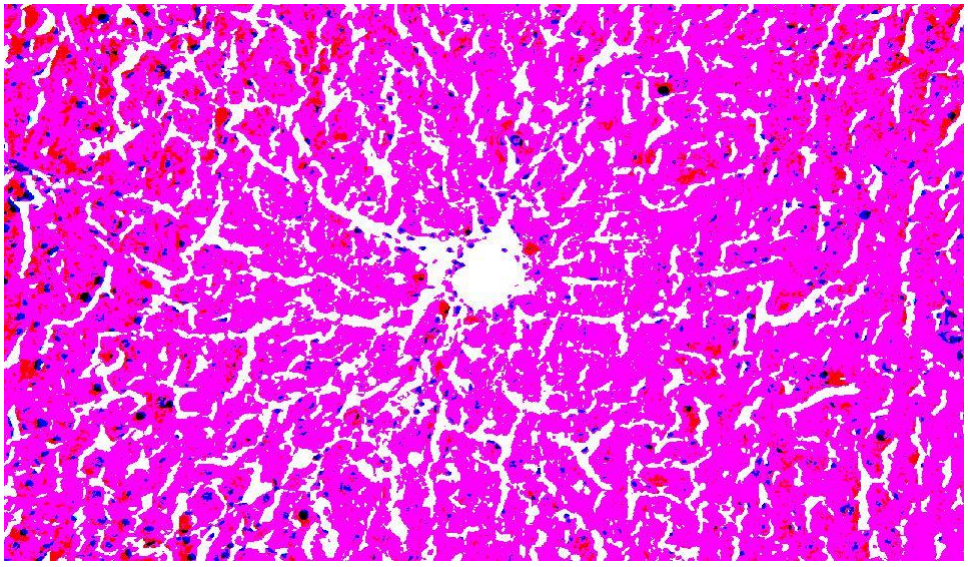

Figure 1E. Photomicrograph of the liver section from DAS-treated NDEA rats (X = 100) showing more or less normal hepatic lobule architecture.

NO radical is considered to exert a hepatoprotective action against tissue injury and cytotoxic agents. However, any situation that causes uncontrolled, prolonged and/or massive production of NO, by the inducible NO Synthase (iNOS) may result in liver damage, leading to inflammation and even tumor development. The harmful effects of NO radical may be attributed to its reaction with superoxide anion to yield peroxynitrite, which is a potent oxidizing and nitrating agent. Peroxynitrite can oxidize nuclear DNA and membrane phospholipids and can nitrate free or protein-associated tyrosines and other phenolics (Inoue and Kawanishi, 1995; Natal et al., 2008). In our study, increased level of NO was observed in liver tissue of NDEA-treated rats. Manjeet and Ghosh (1999) have been shown that iNOS, which is stimulated in liver of rats treated with NDEA, is regulated at the transcriptional level by cytokines and by the exposure of cells to other inflammatory stimuli such as endotoxin or ROS.

iNOS may contribute to tumor promotion via NO radical production and subsequent action of peroxynitrite. NO radical contributes to cell differentiation, which may provide a molecular basis for abnormal differentiation of hepatocytes in hepatocellular carcinomas and/or biliary epithelial tumors (Vasil'eva et al., 1997). Treatment of tumor bearing rats with DAS resulted in a significant decrease in liver NO production. Chang and Chen (2005) showed that DAS inhibit the enhanced NO production by decreasing iNOS expression and by its direct NO clearance activity.

Tumor bearing rat liver showed increased levels of GSH and PSH contents along with increased activity of GR, GPX, GST, GCS, GT and G6PD enzymes. GSH associated enzymes play vital role in determining sensitivity to anticancer drugs. The capacity of a tumor cell to maintain GSH is determined by a number of interacting pathways. Many of the enzymes involved in these pathways have been targeted for therapeutic intervention by modulators of anticancer drug resistance. Increase in the expression of GST in multi -drug resistance cell lines has frequently been cited as a causal resistance mechanism (Tew, 1994). Increased expression of GCS, the rate-limiting enzyme in de novo synthesis of GSH, along with GT may be causally linked to resistance to alkylating agents and platinum-based anticancer drugs (Goodwin et al., 1992). In addition, increased activity of GR has been observed in tumor bearing group, functioned to recycle GSH with subsequent increase in the resistance against chemotherapeutic drugs. The decreased activity of these enzymes in the DAS treated group administered NDEA is indicative of its ability to inhibit tumor progression. G6PD, a house-keeping enzyme catalyzes the first step in the hexose monophosphate pathway, produces ribose, which is incurporated into nucleotides and NADPH, the major cytoplasmic reducing compound. NADPH is necessary for reduction of oxidized glutathione by GR and is a sub-strate for phase I and II detoxification enzymes. G6PD is elevated in response to external stimuli like toxic agents and oxidative stress. Frederiks et al. (2003) found that the activity of G6PD is up regulated by carcinogens and oxidative stress. DAS administration to NDEA-treated rats showed elevated G6PD activity, indicating that increased amounts of NADPH are required for detoxi-fication process.

The present study revealed that the levels of lactate; pyruvate and lactate/pyruvate ratio along with LDH activity didn't significantly change in the rats with NEDA-induced hepatic cancer. These results are coinciding with the finding of Sauer and Dauchy (1985) using hepatoma 5123C cells. It was believed for decades that tumors are high lactate producers until the report of Sauer and Dauchy (1985) who indicated that tumors *in vivo* have a large capacity for lactate utilization as well as for its production. However, liver ATP level was significantly decreased in such group of rats. This fall in ATP level could be attributed to the high level of NO observed in that group. Nishikawa et al. (1996) demonstrated that NO reversibly inhibits both respiration and ATP synthesis in ascites hepatoma AH130 cells. Consistent with this notation is the observation that NO interacted with cytochrome c oxidase and inhibited the respiration of rat liver mitochondria (Cleeter et al., 1994). Alternatively, in addition to the inhibition of ATP synthesis NO might possibly enhance the ATP utilization by cells (Nishikawa et al., 1996). Independently of the mechanism by which the ATP synthesis is down-regulated in liver carcinomas, it is reasonnable to suggest that the low liver mitochondrial cellular content could contribute to the expansion of cancer cells and, perhaps, to their resistance to chemo- and radiotherapy, because the overall oxidative phosphorylation capability of the cell is diminished. Thus, the apoptotic potential of the cancer cell is hampered (Dey and Moraes, 2000). Previous studies reported that liver carcinogenesis is accompanied by repression of the program of mito-chondrial biogenesis that is responsible for the prolife-ration of mitochondria in the hepatocyte (Cuezva et al., 2002). DAS administration to NDEA-treated rats resulted in significant decrease in hepatic lactate level and lactate/ pyruvate ratio with significant increase in pyruvate and LDH activity. Increase in hepatic pyruvate level with de-crease in lactate level along with increase in LDH activity directs glycolysis to aerobic pathway to produce ATP. These changes also may be as a result to the observed decrease in NO level observed in such group of rats.

Conclusion

In conclusion, DAS could potentially attenuate the hepatocarcinogenesity induced by NDEA as evidenced by:

i.) Restoration of almost all enzymatic and non-enzymatic liver function tests.
ii.) Normalization of the oxidative stress biomarker, MDA, along with attenuation of NO.
iii.) Balancing of the oxidant/antioxidant (both non-enzymatic and enzymatic) status of the liver cells.
iv.) Improving the energy metabolism and aerobic conditions of the hepatic tissue.
iv.) Recovery of the histopathological changes shown in NDEA–administered rats.

Thus, it is recommended to consume moderate level of garlic, a natural source of DAS, in a normal diet, which can inhibit the activation of some types of environmenttally encountered procarcinogens. Future researches are recommended to shed more light on the unstudied biochemical properties of DAS, and related compounds, on both cellular and molecular levels. This will aid in the development of potent; naturally occurring, or synthetic, anticarcinogenic agents.

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