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Impact of hypercholesterolemia on toxicity of N-nitrosodiethylamine: biochemical and histopathological effects

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Abstract:
N-nitrosodiethylamine (NDEA) is an important carcinogen frequently present in human environment and food chain. Nitrosamines such as NDEA produce oxidative stress due to generation of reactive oxygen species and alter the antioxidant defence system in the tissues. The present investigation was aimed at studying its toxicity under hypercholesterolemic conditions. NDEA administration brought about hepatic degeneration as evidenced by the significant decrease in liver weight index of both normal as well as hypercholesterolemic animals. Hypercholesterolemia did not affect the hemoglobin (Hb) content in experimental animals but resulted in an increase in the osmotic fragility of erythrocytes. The antioxygenic potential of experimental animals decreased in both, the NDEA-fed group as well as in the group that was also supplemented with a hypercholesterolemic diet. This was evident by increased *in vitro* lipid peroxidation (LPO) of erythrocytes. Administration of NDEA resulted in a substantial and significant increase in LPO in all the tissues under normal as well as hypercholesterolemic conditions. Addition of hypercholesterolemic diet in general, increased LPO in all the tissues to varying degrees but its effect was maximal in the liver. Effect of NDEA administration on antioxygenic enzymes under normal as well as hypercholesterolemic conditions was variable in different tissues. Histopathological analysis of different tissues (heart, liver, lungs, spleen and kidneys) showed mild to severe pathological changes among the control and experimental groups.

Key words:
NDEA, lipid peroxidation, hypercholesterolemia, antioxygenic potential

Abbreviations: ALT – alanine aminotransferase, AST – aspartate aminotransaminase, LDL – low density lipoprotein, LPO – lipid peroxidation, NOCs – N-nitroso compounds, NDEA – N-nitrosodiethylamine, NDMA – N-nitrosodimethylamine, ROS – reactive oxygen species, SOD – superoxide dismutase, TBARS – thiobarbituric acid reactive substances

Introduction

N-nitroso compounds (NOCs) are one of the important groups of carcinogens frequently present in human environment and food chain [28]. Presence of nitroso compounds, like N-nitrosodimethylamine, N-nitrosodiethylamine, N-nitrosopyrrolidine and N-nitrosopiperidine has been widely reported in various foodstuffs, such as milk products, meat products, soft drinks and alcoholic beverages [18, 27, 33, 34]. Besides this, NOCs are readily formed in human body by the reaction of nitrite with amines and amides [21, 25]. The presence of nitroso compounds and their precursors in human environment together with the possibility of their endogenous formation in human body have led to suggestions of their potential involvement

in human cancers [6, 15]. Nitroso compounds such as N-nitrosodiethylamine (NDEA) have been suggested to cause oxidative stress and cellular injury due to involvement of free radicals [3–5]. There is considerable support to the concept that oxygen free radicals and related lipid peroxides also play a key role in the pathogenesis of normal senescence and of age-related chronic degenerative diseases, including atherosclerosis [22, 23]. A number of studies indicate a direct correlation between high levels of plasma low density lipoprotein cholesterol (LDL-cholesterol) and atherosclerosis [12, 17]. Production of oxidized LDL in the intima, and removal of oxidized LDL particles by scavenger receptors on macrophages play a central role in the development and progression of atherosclerotic lesions [16]. Although there are several reports where acute doses of NDEA, varying between 100 and 200 mg/kg of body weight were used to show its carcinogenic effects [4, 26, 35], there is no study to show its toxicity under hypercholesterolemic conditions. The present investigation was, therefore, aimed at studying the toxicity of NDEA under hypercholesterolemic conditions and its effect on oxidative stress and antioxidant defence system in the body.

Materials and Methods

All the chemicals used in the present study were of analytical grade. N-nitrosodiethylamine was procured from Sigma Chemical Company, St. Louis Missouri, USA.

Animal experiments were approved by the Social Justice & Empowerment Committee for the purpose of control and supervision of experiments on animals, Ministry of Government of India, New Delhi. Disease free male albino rats (6–8 weeks old) were obtained from the Small Animal Colony of Punjab Agricultural University, Ludhiana. The rats were divided into four groups of 6 rats each. Rats of group I and II were fed on normal diet, whereas rats of group III and IV were given hypercholesterolemic diet containing 1% cholesterol. All the rats were fed on these diets for 21 days before administration of NDEA. Rats of group I and III served as respective controls for group II and IV rats, and were given *ip* injections of normal saline, whereas, group II and IV received *ip* injections of NDEA at 200 mg/kg of body weight (LD_{50} of NDEA). All the rats continued to be fed on their respective diets and were sacrificed 48 h after NDEA administration. Their blood and tissues were collected for biochemical and histopathological analyses.

Blood samples were collected directly by cardiac puncture into tubes rinsed with saturated EDTA. Organs, such as liver, heart, spleen, kidneys and lungs were removed and a portion of these tissues was also stored in 10% formalin for histopathological examination. Blood samples were centrifuged to obtain plasma for biochemical analysis. Erythrocytes were washed with buffered saline (pH 7.4) and a 5% packed cell volume was prepared. A 10% tissue homogenate was prepared in 0.1M phosphate buffer for estimating lipid peroxidation.

Hemoglobin (Hb) and osmotic fragility of erythrocytes were estimated by the methods of Dacie and Lewis [9]. To assess the oxygenic potential of erythrocytes, *in vitro* LPO of erythrocytes was determined by exposing erythrocytes to 40 mM H_2O_2 and by determining the thiobarbituric acid reactive substances (TBARS) produced using thiobarbituric acid, as described by Stocks and Dormandy [31]. Plasma aminotransferases namely, aspartate aminotransferase (AST) (EC 2.6.1.1) and alanine aminotransferase (ALT) (EC 2.6.1.2) were assayed by the method described by Bergmeyer [7]. Lipid peroxidation of tissue homogenates was determined by the method of Ohkawa et al. [24], as modified by Jamall and Smith [14], by malonyldialdehyde-thiobarbituric acid (MDA-TBA) reaction. Superoxide dismutase (SOD) (EC 1.15.1.1) activity was determined by the ability of enzyme to inhibit autooxidation of pyrogallol as described by Marklund and Marklund [20]. Catalase (EC 1.11.1.6) activity was assayed by the decomposition of H_2O_2 as described by Aebi [1]. Peroxidase (EC 1.11.1.7) activity was measured by the method of Claiborne and Fridovic [8].

All data were expressed as the mean ± standard deviation, and Student's *t*-test was used for comparative analysis. The results were considered significant if the p value was 0.05 or less.

Results

Administration of NDEA resulted in substantial decrease in food intake (Tab. 1). However, the effect

Tab. 1. Intraperitoneal toxicity of NDEA (at 200 mg/kg of body weight in groups II and IV) under hypercholesterolemic conditions. Effect on feed intake, change in body weight, organ weight indices and activities of plasma transaminases

Parameter	Dietary condition			
	Normal		Hypercholesterolemic	
	I	II	III	IV
Feed intake/day (g)	11.0 ± 0.81	5.0 ± 0.60[a]	10.0 ± 0.78	8.0 ± 0.75[b]
Change in body weight (g)	0.0 ± 0.0	–(13.0 ± 2.6)[a]	2.0 ± 0.94	–(9.0 ± 2.05)[a]
Specific organ weight				
Heart	0.294 ± 0.009	0.310 ± 0.016[b]	0.376 ± 0.018	0.410 ± 0.008
Lung	0.609 ± 0.05	0.620 ± 0.06[a]	0.789 ± 0.11	0.801 ± 0.09
Liver	4.02 ± 0.11	2.46 ± 0.09[a]	4.98 ± 0.17	3.62 ± 0.26[a]
Spleen	0.196 ± 0.01	0.216 ± 0.02[b]	0.242 ± 0.02	0.267 ± 0.02[b]
Kidney	0.602 ± 0.02	0.634 ± 0.09	0.686 ± 0.08	0.742 ± 0.06
ALT (U/L)	9.10 ± 0.71	96.60 ± 4.02[a]	9.16 ± 0.75	78.00 ± 3.46[a]
AST (U/L)	14.48 ± 0.83	76.66 ± 3.10[a]	14.50 ± 0.59	78.66 ± 3.75[a]

Values are the mean ± SD, n = 6; [a] $p < 0.01$, [b] $p < 0.05$ w.r.t. control

was less pronounced under conditions of hypercholesterolemia. NDEA administration resulted in a loss in body weight in normal as well as hypercholesterolemic rats. The decrease in body weight was smaller in hypercholesterolemic rats.

NDEA administration brought about hepatic degeneration as evidenced by the significant decrease in liver weight index in both normal as well as hypercholesterolemic animals (Tab. 1). Administration of NDEA also resulted in heart enlargement. Spleen weight increased which can be attributed to the chemical intoxication and immunological responses generated against the carcinogen [27]. NDEA administration caused a substantial liver damage as evidenced by nearly 5- and 10-fold increase in the activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) enzymes, respectively. However, the effect of NDEA administration on ALT activity was relatively smaller under hypercholesterolemic dietary conditions. The effect on AST appeared to be similar under both normal and hypercholesterolemic dietary conditions.

Hemoglobin (Hb) content was unaffected by NDEA administration under either of the dietary con-

Tab. 2. Intraperitoneal toxicity of NDEA (at 200 mg/kg of body weight in groups II and IV) under hypercholesterolemic conditions. Effect on Hb, osmotic fragility, *in vitro* LPO and antioxidant enzymes in erythrocytes

Parameter	Dietary condition			
	Normal		Hypercholesterolemic	
	I	II	III	IV
Hb (g/dL)	14.62 ± 1.10	14.65 ± 0.97	12.87 ± 1.02	11.82 ± 2.15
Osmotic fragility (% hemolysis)	26.07 ± 1.90	27.50 ± 1.70	41.60 ± 3.16	43.87 ± 2.45
LPO^	570.30 ± 47.70	572.80 ± 41.30	683.40 ± 37.30	813.70 ± 73.9[a]
Catalase*	101.60 ± 12.10	96.10 ± 14.60	84.57 ± 10.90	75.90 ± 8.61
Peroxidase**	1.92 ± 0.28	1.86 ± 0.10	0.69 ± 0.06	1.01 ± 0.37
SOD***	2.26 ± 0.29	1.87 ± 0.21[b]	2.59 ± 0.70	3.33 ± 0.57

Values are the mean ± SD, n = 6; [a] $p < 0.01$, [b] $p < 0.05$ w.r.t. control; ^ = n moles of MDA formed/g Hb/h; * Expressed as standard international units; ** 1 unit = increase in 1 O.D./min; *** 1 unit = amount of enzyme that inhibits 50% of auto-oxidation of pyrogallol

ditions (Tab. 2). Osmotic fragility of the erythrocytes was marginally increased by NDEA administration, the effect being more pronounced under hypercholesterolemic conditions. The increased osmotic fragility of erythrocytes is supported by the observed similar increase in the *in vitro* lipid peroxidation (LPO) of erythrocytes (Tab. 2).

NDEA administration also resulted in a substantial and significant increase in LPO in all the tissues under normal as well as hypercholesterolemic conditions (Tab. 3), and the effect was relatively stronger under normal dietary conditions, except in the heart and kidneys. Hypercholesterolemia in general increased the LPO in all the tissues through varying degrees but its effect was maximal in the liver.

Moreover, the activities of antioxidant enzymes were also affected in response to NDEA administration differently in different tissues. In general, NDEA administration induced a marginal decrease or no change in catalase and SOD activities. However, the activity of peroxidase significantly increased in response to NDEA administration in both normal as

Tab. 3. Intraperitoneal toxicity of NDEA (at 200 mg/kg of body weight in groups II and IV) under hypercholesterolemic conditions. Effect on lipid peroxidation and activities of antioxidant enzymes in different tissues

Parameter	Dietary condition			
	Normal		Hypercholesterolemic	
	I	II	III	IV
		Heart		
LPO^	91.12 ± 8.50	141.00 ± 12.80[a]	96.45 ± 6.40	162.20 ± 5.19[a]
		Antioxidant enzymes[#]		
Catalase*	31.26 ± 3.70	33.50 ± 3.50	75.57 ± 3.23	74.11 ± 6.60
Peroxidase**	0.468 ± 0.06	0.648 ± 0.06[a]	0.496 ± 0.03	0.530 ± 0.03
SOD***	10.59 ± 1.60	11.01 ± 1.30	14.61 ± 2.03	19.69 ± 2.62[a]
		Lung		
LPO^	123.30 ± 11.00	161.20 ± 15.29[a]	241.88 ± 17.07	254.70 ± 24.37
Catalase*	47.54 ± 4.30	38.25 ± 3.70[a]	65.39 ± 2.93	49.82 ± 5.93[a]
Peroxidase**	0.529 ± 0.06	0.621 ± 0.06[b]	0.873 ± 0.10	1.120 ± 0.27[b]
SOD***	17.39 ± 3.20	15.30 ± 2.20	28.78 ± 2.62	25.75 ± 2.62[a]
		Liver		
LPO^	194.20 ± 18.80	247.10 ± 21.30[a]	323.93 ± 21.80	349.57 ± 16.30[b]
Catalase*	363.10 ± 50.00	212.00 ± 28.10[a]	336.70 ± 45.70	168.30 ± 34.10[a]
Peroxidase**	0.167 ± 0.02	0.432 ± 0.04[a]	0.246 ± 0.02	0.440 ± 0.06[a]
SOD***	9.39 ± 0.51	8.31 ± 1.10[b]	17.38 ± 1.37	16.59 ± 1.37
		Spleen		
LPO^	228.30 ± 17.90	300.90 ± 26.70[a]	274.00 ± 18.53	339.90 ± 26.38[a]
Catalase*	51.00 ± 5.30	52.60 ± 5.10	47.43 ± 3.43	54.59 ± 8.33
Peroxidase**	0.789 ± 0.10	0.772 ± 0.06	1.22 ± 0.28	1.09 ± 0.24
SOD***	9.60 ± 1.10	9.49 ± 1.00	14.10 ± 1.46	12.98 ± 0.48
		Kidney		
LPO^	225.90 ± 19.65	276.60 ± 23.40[a]	262.40 ± 16.18	321.40 ± 25.93[a]
Catalase*	288.00 ± 19.10	206.10 ± 20.50	250.00 ± 24.64	207.00 ± 5.82[a]
Peroxidase**	0.219 ± 0.03	0.457 ± 0.05[a]	0.440 ± 0.05	0.513 ± 0.07
SOD***	11.49 ± 1.50	9.61 ± 1.10[b]	19.09 ± 1.07	17.60 ± 0.50[a]

Values are the mean ± SD, n = 6; [a] $p < 0.01$, [b] $p < 0.05$ w.r.t. control; ^ = n moles MDA formed/g tissue; # = Units/mg protein; * Expressed as standard international units; ** 1 unit = increase in 1 O.D./min; *** 1 unit = amount of enzyme that inhibits 50% of auto-oxidation of pyrogallol

well as hypercholesterolemic conditions. The effect on antioxygenic potential was relatively more pronounced under normal dietary conditions.

Histopathological examination of tissues under light microscope was done to observe the effect of NDEA on the structural integrity of the cells in heart, lung, liver, spleen and kidney, under hypercholesterolemic conditions. Hypercholesterolemia caused mild degenerative changes but administration of NDEA caused remarkable cellular degeneration in these organs. In heart, hypercholesterolemic conditions resulted in mild hemorrhage and edema in–between cardiac muscle fibers. However, administration of NDEA under hypercholesterolemic conditions resulted in severe accumulation of lipid droplets in myocardium fibres along with degenerative changes. In lungs, interstitial pneumonia was observed along with infiltration of mononuclear cells under hypercholesterolemic conditions but administration of NDEA resulted in chronic interstitial pneumonia along with infiltration of leukocytes. Under hypercholesterolemic conditions, liver showed vacuolar degeneration and swelling of hepatocytes but more severe changes were seen upon NDEA administration, such as accumulation of lipid droplets in hepatocytes, granular degeneration along with infiltration of fibroblasts indicating chronic change. In spleen, there was a depletion of lymphocytes from lymphoid follicles under hypercholesterolemic conditions. Administration of NDEA, however, also resulted in congestion and hemorrhage in spleen along with severe depletion of lymphoid cells. Kidneys showed mild congestion under hypercholesterolemic conditions but NDEA administration caused severe granular degeneration and coagulative necrosis in kidneys.

Discussion

Nitrosamines are known carcinogenic agents. However, their mechanism of action is not well understood. Generation of reactive oxygen species could be an important cause of toxicity of nitrosamines [5, 21]. These cellular prooxidant states can initiate oxidation of lipids and other biomolecules [11, 30]. Biological molecules can lead to alkylation of genetic material and proteins associated with this material. Nitrosamines such as N-nitrosomorpholine (NMOR) have been shown to induce DNA damage *via* direct formation of reactive oxygen or nitrogen species (ROS/RNS) [29]. Excess production of these ROS/RNS, their production in inappropriate relative amounts (especially superoxide and NO) or deficiencies in antioxidant defences may result in pathological stress to cells and tissues. Considerable evidence supports the view that oxidative damage involving both ROS and RNS is an important contributor to the development of atherosclerosis [10, 13]. Peroxynitrite (derived by reaction of superoxide with nitric oxide) and transition metal ions (perhaps released by injury to the vessel wall) may contribute to lipid peroxidation in atherosclerotic lesions [10]. Metabolic activation of NDEA possibly produces ROS capable of initiating damage in the cell. The increased oxidative stress may alter the antioxidant system or the metabolic products of NDEA interfere with the antioxidant system and, hence, increase LPO. Taniguchi et al., [32] have similarly studied the effects of N-nitrosodimethylamine (NDMA), particularly on the oxidative status of rat liver. They have shown that at lower dose levels of NDMA, protective responses were prominent, such as elevation of the hepatic glutathione and metallothionein (MT) levels, and increase in the activities of gamma-glutamylcysteine synthetase, glucose-6-phosphate dehydrogenase (G6PD), and malic enzyme. This finding corroborates our result wherein there is an increase in the activity of peroxidase in the NDEA-challenged rats, which could be a result of their adaptation to NDEA induced stress. The increased LPO, in spite of increased unaltered activities of defense enzymes, indicates that NDEA administration increases the generation of ROS, as also shown by Taniguchi et al., [32] for high doses of NDMA, which too caused increased lipid peroxide levels in liver and serum, in addition to the increase in glutamic-oxaloacetic transaminase (GOT), glutamic-pyruvic transaminase (GPT), and ketone bodies levels in serum. Ahotupa et al., [2] have, however, also reported that NDMA induced LPO in the target tissue, and slightly decreased antioxidant enzyme activities and GSH contents in the liver. Kaplan et al., [15] have carried out studies aimed at assessing the influence of nutritional factors, including N-nitroso compounds, in the etiology of brain tumors, specifically gliomas and meningiomas and suggested the presence of an interaction between the effects of N-nitroso compounds and cholesterol intake. The data suggest that dietary factors may play an important, though yet undefined,

role in the development of brain tumors. Lu and Chiang [19] have also investigated the effect of dietary fat levels on lipid peroxidation and the activities of antioxidant enzymes in rats. They have found that rats fed high-fat diets showed increased serum and liver thiobarbituric acid reactive substances (TBARS). Moreover, the hepatic catalase activity was found to be lower in rats fed cholesterol-containing diets. These results corroborate our findings. However, at high levels of cholesterol, the oxidative stress also increases and consequently could add to the NDEA-induced damage. The lower degree of effect under hypercholesterolemic conditions in our study can also be attributed to the already higher levels of peroxidative damage owing to NDEA administration, making it to the limiting stage. Histopathological examination showed almost similar degrees of effect of NDEA administration under normal or hypercholesterolemic conditions. Histopathological aberrations could be the result of increased oxidative stress resulting from the administration of NDEA.

Our results suggest an association between the observed changes in biochemical parameters, notably oxidative stress, due to hypercholesterolemia on the toxicity of NDEA. However, the underlying mechanisms involved at the molecular level need to be further explored.

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