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Hepatoprotective effect of food preservatives (butylated hydroxyanisole, butylated hydroxytoluene) on carbon tetrachloride-induced hepatotoxicity in rat

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ABSTRACT

Carbon tetrachloride (CCl_4), a hepatotoxic agent is widely used to study the toxic mechanisms in experimental animals. This study was carried out to establish the hepatoprotective measures of food preservative antioxidants butylated hydroxyanisole and butylated hydroxytolune (BHA, BHT) when mixed with food towards carbon tetrachloride (CCl_4) intoxication (230 mg/ kg b wt/rat/day) in rat. Biochemical markers like serum glutamate pyruvate tranaminase (AST), serum glutamate oxaloacetate transaminase (ALT), alkaline phosphatase (ALP) and bilirubin content, antioxidant enzymes such as SOD, CAT, GPx, and malondialdehyde (MDA) as the end product of lipid peroxidanion were measured. The results had shown the elevated level of AST (121.16%), ALT (124.68%), ALP (122.41%) an, bilirubin content (57.14%) after CCl_4 treatment. Marked decrease of activity of antioxidant enzymes such as SOD (85.36%), CAT (67.47%), GPx (50.7%) had indicated that the ROS mediated toxicity and pretreatment of BHA and BHT restored the activity of these enzymes. High level of MDA content with reduced GSH value was also observed due to oxidative stress. The hepatic antioxidant status was restored with the food preservative (BHA, BHT) antioxidant treatment which had indicated the significant protective effect against CCl_4 induced hepatotoxicity and finally confirmed by histopathological studies.

1. Introduction

The liver is a vital organ, located in the upper right quadrant of the abdomen below the diaphragm and has a wide range of functions related to metabolism of carbohydrate, protein, lipid and xenobiotics which include gluconeogenesis, glycogenolysis, urea biosynthesis, production of plasma proteins and blood clotting factors, cholesterol biosynthesis, production of triglycerides and bile in addition to detoxification of various metabolites. Reactive oxygen species (ROS) resulting from oxidative stress (OS) are mainly by product of normal cellular metabolism. Altered cellular activities of electron transport system (ETS), cyclooxygenase, oxidases, peroxidases are main factors for production of increased amount of ROS due to an increased OS [1]. Liver is frequently exposed to a variety of xenobiotics, pesticides, organic solvents, anasthetics, and drugs. Carbon tetrachloride (CCl_4) is a xenobiotic compound which produces hepatotoxicity in human beings and animals [2]. It appears in the environment specially in the water of industrial wastes from the manufacturing sector of chlorofluorocarbons, dry cleaning fluids, fire extinguishing agents, etc [3]. Cytochrome p450 enzymes (mostly CYP2E1) of endoplasmic reticulum start its metabolism within the body and generate highly reactive trichloromethyl radical ($CCl_3\cdot$) which rapidly reacts with oxygen to form the highly reactive trichloromethylperoxyl radical ($CCl_3OO\cdot$) [3]. The later molecule rapidly reacts with lipids (particularly PUFA) to form lipid peroxidation products. The free radical mediated lipid peroxidation is one of the main mechanisms of hepatic injury by CCl_4 [4,5]. Butylated hydroxyanisole (BHA) and butylated hydroxyl toluene (BHT) are GARS grade phenolic food preservative; are the structural analog of Vitamin E and most widely applied as synthetic antioxidants. They are mostly used in processed foods like, butter, meat, cereals, chewing gum, baked goods, snack foods, beer etc [6]. BHA, BHT have potential role to inhibit lipid peroxidation (LPO) and OS in many experimental models by restoring the cellular antioxidant enzyme status. Thus the present study was designed to investigate the hepato-protective effect of BHA and BHT on CCl_4 induced oxidative stress.

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2. Material and methods

2.1. Chemicals

Carbon tetrachloride, Tris buffer, Sodium chloride (NaCl), Triton-X 100, Potassium dihydrogen phosphate (KH_2PO_4), Dipotassium hydrogen phosphate (K_2HPO_4), Ethylene diamine tetra acetate (EDTA), Sodium hydroxide (NaOH), Chloroform, Trichloro acetic acid (TCA), Thiobarbituric acid (TBA), Potassium hydroxide (KOH), Methanol, Alcohol, and other chemicals were procured from Merck Ltd., SRL Pvt. Ltd., Mumbai, India. 5′, 5′-dithio (bis)-2- nitrobenzoic acid (DTNB), standard reduced glutathione (GSH), were procured from Sigma (St. Louis, MO, USA). All other chemicals were from Merck Ltd., SRL Pvt., Ltd., Mumbai and were of the highest purity grade available.

2.2. Selection of animals and care

The study was conducted on 24 healthy, Wister strain male albino rats (Supplied by CPSEA, Govt. of India registered firm) having a body weight of 100 ± 15 g. These experimental animals were acclimatized in laboratory condition for period of 2 weeks prior to the experiment. Experimental animals were housed three rats/cage in a room with temperature 22 ± 2 °C with 12–12 h dark–light cycles by the side of a humidity of 50 ± 10%. Standard diet and water *ad libitum* were provided to them. The experimental animal care was provided according to the Guiding Principle for the Care and Use of Animals [7]. To carry out the experiments the rats were divided into four equal groups (n = 6/gr) namely, Group I or control (feed normal diet with water *ad libitum*), Group II or CCl_4 induced hepatic dysfunction (normal diet, water *ad libitum* + subcutaneous injection with (CCl_4 at the conc. of 230 mg/kg body wt/rat/day diluted in olive oil) [8], Group III or CCl_4 with pre-treatment by BHA [6] (pre-treated with 0.5 mg/kg BHA mixed with normal diet + subcutaneous injection with CCl_4), and Group IV or CCl_4 with pre- treatment by BHT [6] (pre-treatment of 0.8 mg/kg BHT along with normal diet + subcutaneous CCl_4 injection).

2.2.1. Sacrifice of animals and collection of blood and tissues

This experimental schedule for evaluation of protective function of selected antioxidants was continued for 28 days, after that the animals were sacrificed and blood was collected from the aorta, then liver and kidneys were collected for different biochemical and histological studies. The tissues were stored into −80 °C until preparation of tissue homogenates. For histological examination, liver and kidneys were preserved in 10% neutral formaldehyde solution till processed.

2.2.2. Separation of serum and homogenization of liver and kidney

Serum was separated by centrifugation (1500 × *g*, 15 min) of blood samples and was kept (–80 °C) [9] for the biochemical estimation of different parameters. Tissue homogenates were prepared through the following process; 1.5 g hepatic tissue was washed initially in 0.9% saline followed by immediate homogenization in the ice-cold buffer (0.25 M sucrose, 1 mM EDTA, and 1 mM Tris-HCl, pH 7.4) and then through centrifugation (600 × g, 10 min at 4 °C). Later on, the supernatant was stored (–80 °C) for the biochemical estimation of different parameters [10].

2.3. Biochemical determinations

2.3.1. Biochemical markers of hepatotoxicity

Serum hepatic marker enzymes namely, serum glutamate oxaloacetate transaminases (AST), serum glutamate pyruvate transaminase (ALT) [11], alkaline phosphatase (ALP) [12], LDH [15], bilirubin [13], total protein [14] concentration were measured by using assay kits Sigma (USA). The extent of hepatocytes necrosis was determined by these activities as markers.

2.3.2. Oxidative stress Profile

To evaluate the degree of cellular damage in hepatocytes, lipid peroxidation (LPO) in tissue homogenate was measured by estimating the formation of thiobarbituric acid reactive substances (TBARS). Tissue homogenate was mixed with 20% TCA (1.5 ml) and 1.34% TBA (1.5 ml) then boiled (30 min) and cooled, followed by an addition of 2.5 ml butanol. The mixture was centrifuged for 5 min in 2000 × *g*. Then optical density of the supernatant was measured at 535 nm. TBARS as malondialdehyde (MDA) content of the sample was calculated by using the molar extinction coefficient $1.43 \times 10^{-3} M^{-1} Cm^{-1}$ and expressed as nmol of MDA formed/mg protein [16].

The estimation of GSH from tissue homogenate was done by the method of Ellman. The reaction mixture contained 25% of TCA and then centrifugation (2,000 × g, 15 min); supernatant was diluted to 1 mL with 0.2 M sodium phosphate buffer (pH 8.0) followed by addition of 2 mL DTNB (0.6 mM). After 10 minutes incubation at room temperature, the optical density of the yellow-colored complex formed by the reaction of GSH and DTNB (Ellman's reagent) was measured at 405 nm. The levels of GSH were expressed as μg of GSH/mg protein [17].

2.3.3. Antioxidant Enzyme Profile

Activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) were assayed from hepatic tissue homogenate for assessment of cellular antioxidant enzyme status.

SOD activity of liver and kidney homogenate was determined from its ability to inhibit the auto-oxidation of pyrogallol according to Mestro and McDonald et al. [18]. The samples were measured at 420 nm at 25 °C for 3 min. SOD activity was expressed as unit/mg protein.

CAT activity of these tissue homogenate was measured by the method of Luck [19]. Catalase activity was calculated by using the molar extinction coefficient of $43.6\ M^{-1}cm^{-1}$for H_2O_2. The level of CAT was expressed as unit/mg protein.

The GPx activity of liver and kidney homogenate was evaluated by the method of Paglia and Valentine [20]. Absorbance at 340 nm was recorded for 5 min. Values were expressed in nmol of NADPH oxidized to NADP in per min/mg protein by using the extinction coefficient of $6.2 \times 103\ M^{-1}cm^{-1}$ at 340 nm.

2.3.4. Histological Study

Histological analysis of liver and kidney tissue of each experimental Group was performed by the method of Iranloye and Bolarinwa [21]. The animals were sacrificed and the tissues were immediately perfused in 0.9% saline and formalin, and were fixed for 7 days in 10% formaldehyde. After that, dehydration was carried out in ascending grade of alcohol (70%–100%). To remove the alcohol, the tissues were kept in xylene overnight. Then embedding and casting in paraffin wax with wooden block was completed and sectioning of 5 μm thick was carried out by using a microtome. The sectioned tissues mounted on slides using a thin film of egg albumen smeared on each side. After deparaffinization by using xylene, the sections passed through alcohol, stained with haematoxylin-eosin, and mounted in neutral DPX medium. The slides were then evaluated for pathological changes under microscope.

2.4. Data analysis

The data were calculated and statistical analyses were done by using a statistical package, Origin 6.1, Northampton, Mass, USA. The statistically calculated data were expressed as mean ± SEM, $n = 6$. Comparisons were done between the means of control and CCl_4 administered group, by one way ANOVA, $P < 0.05$, level of significance.