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# Antioxidant effect of butylated hydroxytoluene on ferric nitrilotriacetate induced renal hyper proliferation and injury in rats

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ABSTRACT

This study was designed to investigate the effect of butylated hydroxy toluene (BHT), a phenolic antioxidant used in foods, cosmetics and pharmaceutical products, on Fe-NTA-induced nephrotoxicity in rats. Fe-NTA (alone) treatment enhances ornithine decarboxylase activity to 5.3-fold, and [$^3$H] thymidine incorporation in DNA to 3.5-fold compared with the corresponding saline treated control. The enhanced ornithine decarboxylase activity and DNA synthesis showed a reduction to 2.12–2.15-fold respectively at a higher dose of 2 mg BHT/day/animal, compared with the Fe-NTA treated group. Fe-NTA treatment also enhanced the renal microsomal lipid peroxidation to 2.0-fold and decreased the activities of glutathione and antioxidant enzymes to a range of 2.2–2.5-fold in kidney. These changes were reversed significantly in animals receiving a pretreatment of BHT. Present data suggests that BHT can prevent the toxic effects of Fe-NTA and can serve as a potent chemopreventive agent to suppress oxidant-induced tissue injury and nephrotoxicity in rats.



## 1. Introduction

Fe-NTA is a known potent nephrotoxic agent and induces apoptosis in mouse renal proximal tubules (Kawabata et al., 1997). The major target organ for Fe-NTA damage is the kidney, and it induces acute nephrotoxicity and a high incidence of renal cellular carcinoma in Wistar rats (Singh et al., 2004). Fruits, vitamins and other several herbs with diversified pharmacological properties have shown to be a rich source of cancer chemo-preventive agents (Wattenberg and Coccia, 1990; Qi et al., 1999). Most of these agents intervene at the promotion stage of multistage carcinogenesis (Perchellet and Perchellet, 1989). Nevertheless, many such agents having capability to inhibit carcinogenesis both at the initiation and promotion stages possess anti-oxidant and free radical scavenging properties (Cai et al., 1998). The inhibition of Fe-NTA mediated carcinogenicity in mice by nordihydroguairetic acid (NDGA), a plant lignin was shown earlier (Ansar et al., 1999).

It has earlier been reported that the synthetic phenolic antioxidants (e.g. BHT, BHA) when added to human and animal food lower the incidence of cancer caused by chemical compounds. They prevent cancer via interception of harmful free radicals or activating the detoxifying enzymes of the body, inhibiting the formation of ultimately carcinogenic metabolites and their binding to DNA, and modifying the immune response of the organism. Their action is influenced by their own chemical structure, the composition of carcinogen, the strain, sex and age of experimental animals, the tissue upon which they are supposed to act and the time of their administration in relation to the time of the carcinogen exposure (Hirose, 1999a; Hirose et al., 1999b).

When administered prior to or at the time of carcinogen exposure, the phenolic antioxidant BHT was found to be an effective inhibitor of carcinogenesis in several target organs (Smith et al., 1998). BHA, BHT and propyl gallate are also known to inhibit the development of glutathione-S-transferase placental form (GST-P) positive foci i.e., heterocyclic amine-induced carcinogenesis (Hirose, 1999a). Linoleic acid, curcumin and BHT also significantly inhibit microsome-mediated DNA adduct formation of the breast carcinogen, dibenzo (a) pyrene (Smith et al., 1998). BHA is also known to selectively inhibit DNA adducts in the liver but not in mammary cells (Izzotti et al., 1999). Such chemo preventive agents may prove more effective against chemical carcinogens such as Fe-NTA. These chemical agents act by generating free radicals that play a key role in both initiation and promotion stage of carcinogenesis.

Oxygen-driven species formed in living cells by normal cellular metabolism or exogenous sources are thought to play a causative role in biological process such as mutagenesis, carcinogenesis, reproductive cell death and aging (Frenkel, 1992). The toxicity of

*Abbreviations:* BHT, butylated hydroxytoluene; BUN, blood urea nitrogen; CRN, creatinine; EDTA, ethylene diamine tetra acetic acid; Fe-NTA, ferric nitrilotrilotriacetate; $H_2O_2$, hydrogen peroxide; LDH, lactate dehydrogenases; ROS, reactive oxygen species; sGOT, serum glutamate oxaloacetate transaminases; sGPT, serum glutamate pyruvate transaminases; ODC, ornithine decarboxylase.

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$O_2$ and $H_2O$ is thought to result from the transition metal ion-catalyzed conversion (Haber–Weiss reaction) into hydroxyl radical ($OH^{\cdot}$) which reacts with components of DNA at or near diffusion-controlled rates. Iron and copper ions are the most likely candidates *in vivo* for catalyzing the conversion of $O_2$ and $H_2O$ into $OH^{\cdot}$ through redox cycling and play an important role in oxidative injury (Gutteridge et al., 1990). Such redox active iron is generally a weakly chelated form. However, intranuclear iron deposition in hepatocytes and renal tubular cells in rats treated with Fe-NTA were observed by Kondo et al. (1998). Many different types of protein oxidative modification can be induced by reactive oxygen species (ROS). Carbonylation is an irreversible, non-enzymatic modification of proteins. Carbonyl groups are introduced into proteins by a variety of oxidative pathways. ROS can react directly with the protein or they can react with molecules such as sugars and lipids, generating products (reactive carbonyl species) which then react with protein and lead to the formation of protein carbonyl derivatives (Esra et al., 2008; DalleDonne et al., 2003, 2006). Ferric nitrilotriacetate (Fe-NTA) induces acute proximal tubular necrosis as a consequence of lipid peroxidation and oxidative tissue damage that eventually leads to high incidence of renal adenocarcinomas in rodents (Singh et al., 2004).

BHT has been shown to protect against different kinds of injuries and neoplasms involving oxidative stress (Chung, 1999; Williams, 1999; Hirose, 1999a; Hirose et al., 1999b). In present communication, the effect of prophylactic treatment of BHT, a known anti-oxidant on Fe-NTA-induced renal hyper-proliferation and injury has been shown.

## 2. Materials and methods

### 2.1. Preparation of Fe-NTA

The Fe-NTA solution was prepared by the method of Awai et al. (1979)

### 2.2. Animals and treatments

Male albino rats of Wistar strain (four to six weeks old) weighing 125–150 g were used in this study were housed in an air-conditioned room and had free access to pellet diet and water ad libitum. The experimental animals and protocols were approved by the institutional ethical committee.

### 2.3. Study design

For various sets of biological studies different groups of animals were used. A total of 60 rats were used in the study. Thirty rats were used for studying the effect of BHT on Fe-NTA mediated induction of oxidative stress in kidney. Another group of 30 rats were used for studying effect of BHT on Fe-NTA mediated [$^3$H] thymidine incorporation into renal DNA.

Ornithine decarboxylase (ODC) activity induction and assessment of renal function: for studying the effect of BHT on Fe-NTA mediated generation of renal oxidative stress and ODC induction, 30 rats were taken which were divided into 5 groups of 6 rats each. Group I received saline and serves as negative control. Group II received an oral treatment of BHT (only higher dose) and also served as control. Group III received only corn oil (vehicle of BHT) for a period of one week through the gavage. Group IV and Group V received 1 mg and 2 mg/animal/day in 0.2 ml of corn oil of BHT respectively daily for one week orally. Twenty-four hours after the last treatment of BHT or corn oil, the animals of Groups III, IV and V received an i.p. injection of Fe-NTA (9 mg Fe/kg body weight). All the thirty animals were killed 12 h after the treatment of Fe–Fe-NTA by cervical dislocation within a short period of one hour. Just before the killing, blood of these animals was collected in different test tubes from retro-orbital sinus for the estimation of blood urea nitrogen (BUN) and creatinine (CRN). Kidney was collected and processed for ODC activity and estimation of antioxidants including glutathione, antioxidant enzymes-glutathione peroxidase, glutathione reductase, glutathione S transferase, glucose-6-phosphate dehydrogenase and catalase.

#### 2.3.1. Estimation of CRN and BUN

*2.3.1.1. Estimation of creatinine (CRN).* Creatinine was estimated by the alkaline picrate method (Hare, 1950). Protein free filtrate was prepared. To a 1.0 ml of plasma/serum, 1.0 ml of sodium tungstate (5% w/v), 1.0 ml of sulphuric acid (0.6 N) and 1.0 ml of distilled water were added, mixed thoroughly and centrifuged at 800 g for 5 min. The supernatant was added to a mixture containing 1.0 ml of picric acid (1.05% w/v) and 1.0 ml of sodium hydroxide (0.75 N). The absorbance was recorded exactly after 20 min at 520 nm using a spectrophotometer against a reagent blank.

*2.3.1.2. Estimation of blood urea nitrogen (BUN).* Blood urea nitrogen was estimated by diacetyl monoxime method (Kanter, 1975). Protein-free filtrate was prepared. To a 0.5 ml of protein-free filtrate, 3.5 ml of distilled water, 0.8 ml diacetylmonoxime (2%) and 3.2 ml of sulphuric acid–phosphoric acid reagent (reagent was prepared by mixing 150 ml of 85% w/v phosphoric acid with 140 ml of water and 50 ml of concentrated $H_2SO_4$) was added. The reaction mixture was placed in a boiling water-bath for 30 min and then cooled. The absorbance was recorded at 480 nm using a spectrophotometer against a reagent blank.

#### 2.3.2. Preparation of post-mitochondrial supernatant (PMS) and microsomes

Kidneys were quickly removed, perfused immediately with ice-cold saline (0.85% w/v NaCl) and homogenized in chilled phosphate buffer (0.1 M, pH 7.4) that contained KCl (1.17% w/v), using a Potter Elvehjem homogenizer. The homogenate was filtered through a muslin cloth and was centrifuged at 800 g for 5 min at 4 °C in an Eltek Refrigerated Centrifuge (model RC 4100 D) to separate the nuclear debris. The aliquot obtained was centrifuged at 10 500 g for 20 min at 4 °C to obtain PMS, which was used as a source of enzymes. A portion of the PMS was centrifuged to obtain pellet and was considered to be the microsomal fraction suspended in phosphate buffer (0.1 M, pH 7.4) containing KCl (1.17% w/v).

#### 2.3.3. Determination of ornithine decarboxylase (ODC) activity

ODC activity was determined by utilizing 0.4 ml of renal 105,000 g supernatant fractions per assay tube and measuring the release of $14CO_2$ from DL-[1-14C] ornithine according to the method of O'Brien et al. (1975) as described by Athar et al. (1990). Kidneys were homogenized in Tris–HCl buffer (pH 7.5, 50 mM) that contained EDTA (0.1 mM), pyridoxal phosphate (0.1 mM), PMSF (1.0 mM), 2-mercaptoethanol (1.0 mM), dithiothreitol (0.1 mM) and Tween 80 (0.1% w/v), at 4 °C using a polytron homogenizer (Kinematica AGPT 3000). In brief, the reaction mixture contained 400 μl enzyme and 0.095 ml co-factor mixture that contained pyridoxal phosphate (0.32 mM), EDTA (0.4 mM), dithiothreitol (4.0 mM), ornithine (0.4 mM), brig 35 (0.02% w/v) and [$^{14}$C] ornithine (0.05 μCi) in a total volume of 0.495 ml. ODC activity was expressed as pmol $^{14}CO_2$ released/h/mg protein.

#### 2.3.4. Lipid peroxidation

The assay for renal microsomal lipid peroxidation was done following the method of Wright et al. (1981). The reaction mixture, in a total volume of 1.0 ml, contained 0.58 ml phosphate buffer (0.1 M, pH 7.4), 0.2 ml of renal microsome (10% w/v), 0.2 ml ascorbic acid (100 mM) and 0.02 ml ferric chloride (100 mM). The reaction mixture was incubated at 37 °C in a shaking water bath for 1 h. The reaction was stopped by the addition of 1.0 ml trichloroacetic acid (TCA) (10% w/v). Following addition of 1.0 ml thiobarbituric acid (TBA) (0.67% w/v), all tubes were placed in a boiling water bath for a period of 20 min. At the end, the tubes were shifted to an ice-bath and centrifuged at 2500 g for 10 min. The amount of malonaldehyde formed in each of the sample was assessed by measuring the optical density of the supernatant at 535 nm using a spectrophotometer against a reagent blank. The results were expressed as nmol MDA formed/h/g tissue at 37 °C using a molar extinction coefficient of 1.56 M/cm.

#### 2.3.5. Estimation of glutathione

Reduced GSH in kidney was assayed by the method of Jollow et al. (1974). An aliquot of 1.0 ml of renal PMS (10% w/v) was precipitated with 1.0 ml of sulphosalicylic acid (4% w/v). The samples were kept at 4 °C for at least 1 h and then subjected to centrifugation at 1200 g for 15 min at 4 °C. The assay mixture contained 0.1 ml filtered aliquot, 2.7 ml phosphate buffer (0.1 M, pH 7.4) and 0.2 ml DTNB (40 mg/10 ml of phosphate buffer 0.1 M, pH 7.4) in a total volume of 3.0 ml. The yellow color developed was read immediately at 412 nm on a spectrophotometer (Milton Roy Model-21D). The amount of glutathione was expressed as mmol/g tissue.

#### 2.3.6. Assay of antioxidant enzymes

*2.3.6.1. Glucose 6-phosphate dehydrogenase activity.* The activity of glucose 6-phosphate dehydrogenase was assayed by the method of Zaheer et al. (1965). The reaction mixture in a total volume of 3.0 ml consisted of 0.3 ml Tris–HCl buffer (0.05 M, pH 7.6), 0.1 ml NADP (0.1 mM), 0.1 ml glucose 6 phosphate (0.8 mM), 0.1 ml MgCl2 (8 mM), 0.3 ml of renal PMS (10% w/v) and 2.1 ml distilled water. The changes in absorbance were recorded at 340 nm and the enzyme activity was calculated as nmol NADP reduced/min/mg protein using a molar extinction coefficient of 6.223103 M/cm.

*2.3.6.2. Glutathione reductase activity.* Glutathione reductase activity was assayed by the method of Carlberg and Mannervik (1975), as modified by Mohandas et al. (1984). The assay system consisted of 1.65 ml phosphate buffer (0.1 M, pH 7.6), 0.1 ml EDTA (0.5 mM), 0.05 ml oxidized glutathione (1 mM), 0.1 ml NADPH (0.1 mM) and 0.1 ml of renal PMS (10% w/v) in a total volume of 2.0 ml. The enzyme