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BUTYL *p*-HYDROXYBENZOIC ACID INDUCES OXIDATIVE STRESS IN MICE LIVER – AN *IN VIVO* STUDY

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Abstract: Present study focuses on the evaluation of butyl *p*-hydroxybenzoic acid (butylparaben CAS No: 94-26-8) exerted hepatotoxicity in mice. Oral administration of three different doses of butylparaben (40, 20 and 13.33 mg/0.2 mL olive oil/kg b.w./day) for 30 days has resulted in marked increase in lipid peroxidation. The effect was dose-dependent. Biochemical analysis revealed significant ($p < 0.05$) and dose-dependant reduction in non-enzymatic antioxidants such as glutathione and ascorbic acid content. Significant ($p < 0.05$) reduction in enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase, glutathione transferase were also observed in butylparaben treated groups as compared to control. Our findings prove that the oxidative stress induced by butylparaben plays the central role in the toxicity.

Keywords: butylparaben, oxidative stress, hepatotoxicity

Alkyl-esters of *p*-hydroxybenzoic acid (parabens) are having long history of usage as preservatives in food, pharmaceuticals and in cosmetics. Butyl *p*-hydroxybenzoic acid commonly known as butylparaben (BP) belongs to this large class of synthetic chemicals. Parabens are considered safe and extensively used due to its broad spectrum of antimicrobial activity, good pH and heat stability. Pharmacokinetic studies revealed that BP readily gets absorbed from skin and gastrointestinal tract, metabolized by liver, kidney and skin esterases and gets excreted in the form of different conjugates (1). However, there is growing concern about safety of BP as Darbre et al. has demonstrated that a portion of BP can be absorbed and retained in body tissues without hydrolysis by body esterases (2). In *in vitro* and *in vivo* models, BP mimics estrogen activity and act as potential xenoestrogen (3). Studies showed that BP exerts reproductive, developmental as well as teratogenic toxicity in experimental animals (4–7).

Due to this widespread use and building toxicological database a need arises to investigate mechanism of BP-induced toxicity. An *in vitro* study on rat hepatocytes culture has shown a role of depleted ATP, total adenine nucleotide pools and reduced glutathione levels as major causes of the toxicity but none of the study has stated the role of lipid peroxidation (LPO) and antioxidative enzymes in it (8). A selection of particular drug for amelioration of toxicity caused by a toxicant is generally based on the mechanism of toxicity. The purpose of the present investigation is to investigate whether butylparaben exerted toxicity primarily involves its ability to induce oxidative stress.

MATERIALS AND METHODS

Chemicals

Butyl *p*-hydroxybenzoic acid was procured from Hi-Media Laboratories Pvt. Ltd., Mumbai, India and was of analytical grade. All the other chemicals used were of AR grade.

Experimental animals

Inbred adult, healthy female Swiss strain albino mice weighing 30–35 g were obtained from Torrent Research Centre, Bhat, Gandhinagar-382 428, India. Animals were kept in the Animal House of Zoology Department of Gujarat University, Ahmedabad, India under controlled conditions of temperature (25 ± 2°C), 12 h light/dark cycle and relative humidity (50–55%). They were fed with certified pelleted rodent feed supplied by Amrut Feeds, Pranav Agro Industries Ltd., Pune, India and water *ad libitum*. All the experimental protocols

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were approved by the Committee for the Purpose of Control and Supervision of Experiment on Animals (Reg-167/1999/CPCSEA), New Delhi, India. Animals were handled according to the guidelines published by Indian National Science Academy, New Delhi, India (1991).

Experimental Design

Fifty animals were divided equally in five different groups (n = 10). Treatment schedule of the animals was as follows. Animals from group I (untreated control) were kept untreated and given free access to food and water. Group II (vehicle control) animals were treated with olive oil (0.2 mL/day), which has been used as vehicle to dissolve BP. Animals from group III, IV and V received three different doses of BP (13.33, 20 and 40 mg/0.2 mL olive oil/kg b. w./day), respectively, which were $1/30^{th}$ (LD), $1/20^{th}$ (MD) and $1/10^{th}$ (HD) of the LD_{50}. Oral treatments were given to all the animals for 30 days using a feeding tube attached to hypodermic syringe. Animals were sacrificed on 31^{st} day by cervical dislocation and the liver was quickly isolated and blotted free of the blood.

Oxidative stress parameters assessment

Ten percent homogenate of liver tissue was prepared in 0.1 M Tris-HCl (pH 7.4) buffer for the estimation of malonaldehyde (MDA), catalase, glutathione (GSH), glutathione transferase (GST) and protein. The tissue (0.1 g) was homogenized in 10 mL of norit reagent prepared in 6% trichloroacetic acid for estimation of total ascorbic acid content (TAA). For superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and glutathione reductase (GR) 100 mg tissue was homogenized in 5 mL of cold saline, 5 mL of 0.01% digitonin, 2 mL of 0.01% digitonin and in 5 ml of 1% bovine serum albumin (BSA), respectively. Lipid peroxidation in the liver tissue was measured by estimating MDA, an intermediary product by the method of Devasagayam (9). Protein content of the sample was measured by the method of Lowry et al. (10) using bovine serum albumin as standard. TAA was quantified according to the method described by Roe and Kuether (11). Oxidized ascorbic acid combines with 2,4-dinitrophenylhydrazine to form red color complex which can be read colorimetrially. The method described by Moron et al. (12) was used for glutathione estimation, which is based on the reaction with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB). The colored complex formed was read at 412 nm. NADPH-phenazine methosulfate – nitroblue tetrazolium formazan inhibition method was used to measure SOD levels (13). Catalase estimation was done according to the method described by Sinha et al. (14) utilizing hydrogen peroxide as a substrate. GPx activity was measured by the modified method of Pagilla and Valentine (15). GR was assayed by the method of Mavis and Stellwagen (16), which involves conversion of oxidized glutathione to reduced glutathione. The method described by Habig et al. (17) was used to estimate GST, which is based on the utilization of 1-chloro-2,4-dinitrobenzene as substrate.

Statistical analysis

The data were statistically analyzed using one-way analysis of variance (ANOVA) followed by Turkey's *post hoc* test. The results were expressed as the means ± SEM. Statistical significance was accepted with $p < 0.05$. Correlation coefficient was measured to estimate the strength of linear association between two variables. Pearson's correlation analysis was used to find the correlation between lipid peroxidation and other parameters.

RESULTS

Lipid peroxidation

MDA levels of untreated control and vehicle control were found to be almost equal. Figure 1 shows that all three doses (LD – 46.11%, MD – 128.83%, HD – 239.16%) of BP elevate MDA levels in tissue as compared to control indicating high tissue injury caused by lipid peroxidation. The increase is significant ($p < 0.05$) and dose-dependent ($r = 0.937$).

Non-enzymatic antioxidants

No significant difference was observed in TAA and GSH content of control groups (Figs. 2, 3). Total ascorbic acid and glutathione content of liver tissue was lowed significantly ($p < 0.05$) in the groups orally administered with BP. A decrease in TAA content in LD, MD and HD was 11.34%, 27.03%, 41.02%, respectively. For GSH levels, the reduction was found to be 22.22%, 44.53% and 55.74% in LD, MD and HD groups, respectively. The effect was dose-dependent for TAA ($r = –0.969$) and GSH ($r = –0.995$). Maximum reduction was found in group V having the highest dose of BP.

Enzymatic antioxidants

No significant alteration in SOD, CAT, GPx, GR and GST were observed in different control groups. Figures 4–8 show that oral administration of BP for 30 days resulted in significantly ($p < 0.05$)

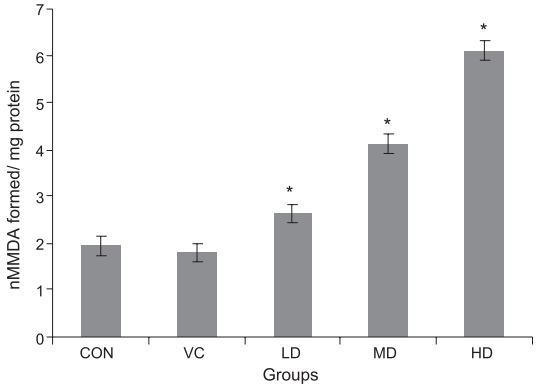

Figure 1. Effect of BP on lipid peroxidation in mice liver. Values are expressed as the mean ± SEM, n = 10; * $p < 0.05$ as compared to the vehicle control group. CON = untreated control, VC = vehicle control, LD = low dose of BP (13.33 mg/0.2 mL olive oil/ kg b.w./day), MD = mid dose of BP (20 mg/0.2 mL olive oil/ kg b.w./day), HD = high dose of BP (40 mg/0.2 mL olive oil/ kg b.w./day)

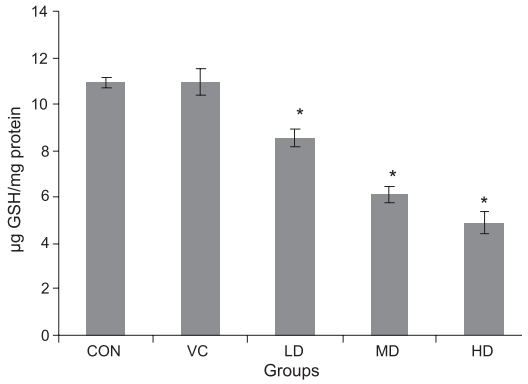

Figure 2. Effect of BP on reduced glutathione (GSH) content in mice liver. Values are expressed as the mean ± SEM, n=10. * $p < 0.05$ as compared to the vehicle control group. Groups marking see Figure 1

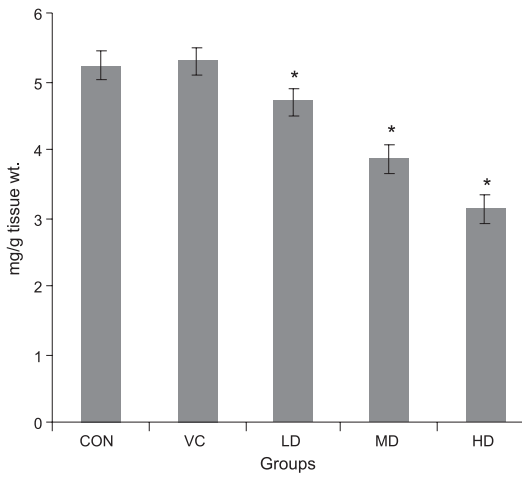

Figure 3. Effect of BP on total ascorbic acid (TAA) content in mice liver. Values are expressed as the mean ± SEM, n = 10. * $p < 0.05$ as compared to the vehicle control group. Groups marking see Figure 1

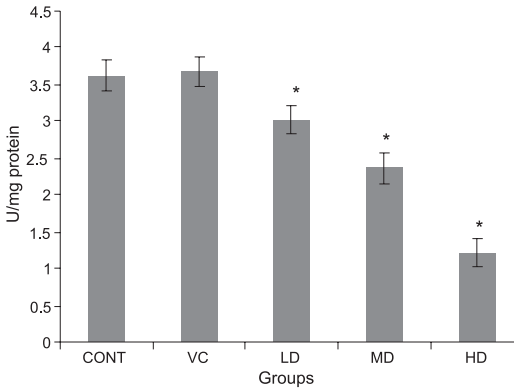

Figure 4. Effect of BP on superoxide dismutase (SOD) in mice liver. Values are expressed as the mean ± SEM, n = 10. * $p < 0.05$ as compared to the vehicle control group. Groups marking see Figure 1

reduced levels of SOD (LD – 17.93%, MD – 35.86%, HD – 66.84%), CAT (LD – 22.24%, MD – 39.43%, HD – 59.25%), GPx (LD – 16.07%, MD – 38.36%, HD –59.34), GR (LD – 12.26%, MD – 33.02%, HD – 48.58%) and GST (LD – 14.03%, MD – 27.06%, HD – 49.32%) as compared to control. Reductions in antioxidant enzyme activity were highly dose-dependent (SOD r = –0.907, CAT r = –0.948, GPx r = –0.969, GR r = –0.980, GST r = –0.915).

Pearson's correlation analysis was used to find the correlation between lipid peroxidation and other enzymatic and non-enzymatic parameters. Strong negative correlation was found between elevated lipid peroxidation and reduced TAA (r = –0.99) and GSH (r = –0.955) levels. Reductions in SOD (r = –0.996), CAT (r = –0.977), GPx (r = –0.991), GR (r = –0.989), GST(r = –0.994) activities were also highly correlated with increased MDA levels of the tissue.

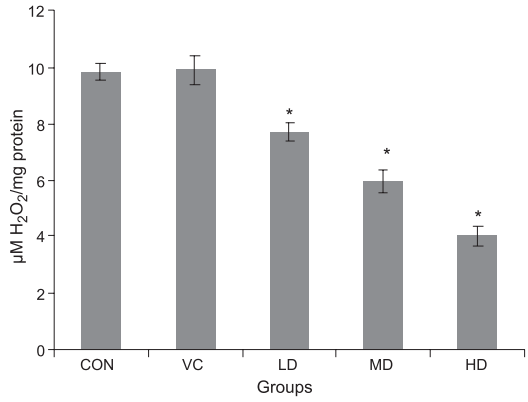

Figure 5. Effect of BP on catalase in mice liver. Values are expressed as the mean ± SEM, n = 10. * $p < 0.05$ as compared to the vehicle control group. Groups marking see Figure 1

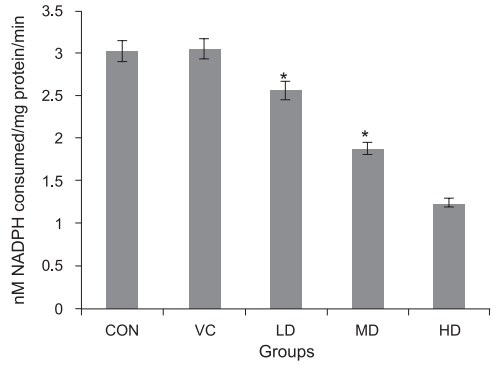

Figure 6. Effect of BP on glutathione peroxidase (GPx) in mice liver. Values are expressed as the mean ± SEM, n = 10. * $p < 0.05$ as compared to the vehicle control group. Groups marking see Figure 1

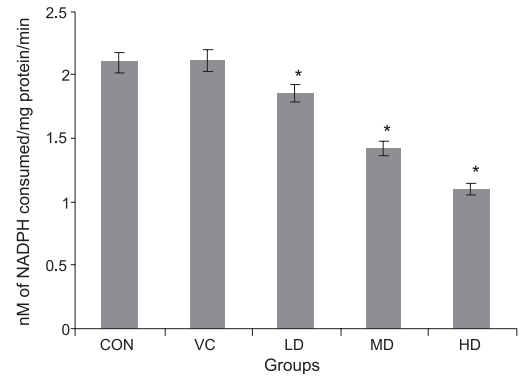

Figure 7. Effect of BP on glutathione reductase (GR) in mice liver. Values are expressed as the mean ± SEM, n = 10. * $p < 0.05$ as compared to the vehicle control group. Groups marking see Figure 1

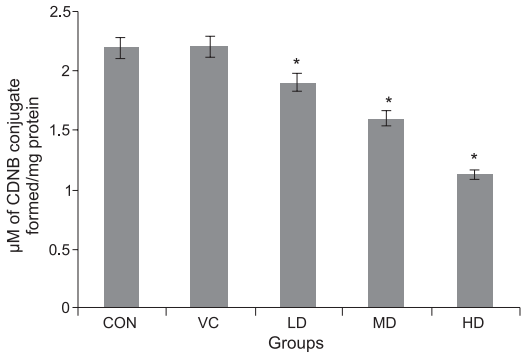

Figure 8. Effect of BP on glutathione-S-transferase (GST) in mice liver. Values are expressed as the mean ± SEM, n = 10. * $p < 0.05$ as compared to the vehicle control group. Groups marking see Figure 1

DISCUSSION

Oxidative stress is a general term used to describe the damage caused by reactive oxygen species (ROS) such as superoxide ($O_2\cdot$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH$). Numerous xenobiotics exert their toxicity by inducing oxidative stress where there is insufficient antioxidant activity leading to excessive accumulation of free radicals, which damage cellular compounds such as proteins, carbohydrates, DNA and lipids (18). Since liver is the prime organ involved in metabolism of these xenobiotics, it is prone to be attacked by the free radicals produced by them resulting in the tissue injury. Free radical damage is generally combated by inbuilt enzymatic and non-enzymatic antioxidant defense systems of body which includes SOD, catalase, GPx, GST, GR, GSH and ascorbic acid.

Findings of the present study clearly indicate the involvement of oxidative stress caused by ROS generation in BP-induced hepatotoxicity. Measurement of MDA levels in the tissue is a great marker of lipid peroxidation which is among the chief mechanism of cell damage. As the carbon numbers of alkyl group attached on paraben increases, water solubility of the compounds decreases. Chemical structure of BP makes it highly lipophilic in nature due to which it can easily penetrate/interact with the lipid membrane of the hepatocytes. Oral administration of

BP for 30 days has resulted in significant elevation in MDA levels which could be due to two possibilities: 1) either due to incorporation of BP moiety itself/overproduced ROS by it in the plasma membrane of cells; or 2) suppression of antioxidative system by BP resulting in altered redox potential of cell causing LPO and hence suggesting a considerable hepatocytic oxidative stress. Administration of *p*-hydroxybenzoic acid, parent component of BP in mice for 30 days resulted in significant increase in liver and kidney LPO levels (19).

Reduced glutathione (GSH) and ascorbic acid are important endogenous free radical scavenger and non-enzymatic antioxidants. The levels of GSH and TAA were significantly reduced in BP-treated animals. This reduction might be due to excessively produced free radicals which crosses the scavenging potency of these antioxidants. Nakagawa and Moldeus (8) had shown the reduction in GSH content in BP exposed isolated hepatocytes, which supports our study. Enzymatic antioxidants include SOD, catalase, GPx and GST, which constitute the first line of defense against ROS-induced damage (20, 21). GR contributes to the regeneration of GSH, so the suppressed activity of GR could be the reason for the reduction of GSH content. The significantly reduced activities of these enzymes might be due to BP-induced protein oxidation. The interaction of LPO products with enzyme molecules leads to the exclusive modification of histidine residue and generation of protein-protein crosslinked derivatives causing reduction in enzyme activity (22). The results of the correlation analysis have shown strong negative correlation between LPO and the activities of enzymes. Reduction in activity of antioxidative enzymes renders free radical scavenging potency of the system.

Thus it can be concluded that treatment of BP for 30 days causes alteration in antioxidative systems as well as increases lipid peroxidation ultimately causing oxidative stress in experimental animals. From this finding of the mechanism further work on the suitable antidote to combat BP toxicity can be designed.

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