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Arsenic and Di(2-ethylhexyl) Phthalate Interact to Alter Individual Effects of some Oxidative Stress Indices in Rat

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ABSTRACT

Arsenic and phthalate are common contaminants found in the environment and concurrent exposure to these agents is highly probable. We therefore, investigated the effects of sodium arsenite (100 mg/l in drinking water) and di(2-ethylhexyl) phthalate (DEHP) (100 mg/kg body weight by gavage) individually and in combination on some oxidative stress indices in the rat. Exposure to either arsenite or DEHP resulted in a significant elevation of advanced oxidative protein products (AOPP) and lipid hydroperoxides (LOOH) concentrations in the plasma, liver, kidney and brain. The combined exposure reflected moderate additive effect on AOPP and LOOH levels in the liver and kidney respectively. The induced increases in the brain AOPP and LOOH contents were significantly more than either single treatment. Exposure to either toxicants alone led to pronounced reduction in the total antioxidative capacity (TAC) in the kidney and brain of the rats. Interestingly however, an antagonistic effect was produced by their interaction in the liver with a reversal of their individual effects. Paraoxonase activity was also drastically inhibited by all treatments in all the compartments studied. Coexposure to arsenite and DEHP displayed some specific interactive effects that are different from individual effects.

Keywords: arsenic, di(2-ethylhexyl) phthalate, coexposure, oxidative stress, total antioxidative capacity,paraoxonase

INTRODUCTION

Exposure of human populations to toxic environmental contaminants is a global health concern and this contamination of the environment is rarely due to a single pollutant. In practice, individuals and populations are generally exposed to a mixture of these chemicals[1,2]. While humans are concurrently exposed to multiple numbers of chemicals and sequentially from different sources through several routes, toxicological studies have focused largely on the evaluation of exposures to single environmental agents[1,3,4]. The paucity of information on the health effects of exposure to complex mixtures of contaminants, therefore, necessitates the need to carry out studies along these areas.

One of the most important environmental agents is arsenic, which according to the Agency for Toxic Substances and Disease Registry, is one of the most hazardous chemicals[5]. Arsenic is a toxic metalloid that is ubiquitous in the environment and is a common contaminant of drinking water. Exposure to arsenic has been linked to multiple adverse health effects with the ingestion of the metalloid increasing risk of cardiovascular disease, hepatic disease, and cancer in millions of people worldwide[6-8].

Phthalates are synthetic chemical agents commonly utilized and found in a variety of industrial products[9]. They are ubiquitous contaminants used as plasticizers, solvents and additives in many consumer products and have been implicated in several diseased conditions[10]. There has been increased report of the potential risk of these chemicals to human health from studies in various species[11] with exposure associated with endocrine disruption[12], metabolic disorders[13], developmental and reproductive defects[14]. Diethylhexyl phthalate (DEHP), the most widely used phthalate derivative, is commonly used as a plasticizer in pharmaceutical and medical devices and also found in cosmetics, as well as in industrial paints and solvents[15,16]. DEHP, like other phthalates, is not chemically bound to the products and thus, has a tendency to leach, resulting in significant environmental contamination and human exposure[17,18].DEHP and its metabolite, mono(2-ethylhexyl)phthalate (MEHP), had been shown to induce testicular damage in animals[19] and decreased sperm motility[20].

Exposure to either arsenic or phthalate is known to induce oxidative damage, a condition which is known to contribute to the associated risk of exposure to these agents[21,22]. Both arsenic and phthalates are found in the air, soil, drinking water and food[6,23]. It is, therefore, not unlikely that there will be incidences of their co-exposure in general populations. Information on the toxicity of their interaction is however, lacking.

The present study aimed to investigate the effects of the combined exposure of inorganic arsenite and the phthalate, DEHP on paraoxonase activity and some oxidative stress indices in tissues of Wistar rats.

Materials and Methods:

Chemicals:

Sodium arsenite and DEHP were purchased from Sigma-Aldrich, Munich, Germany. All other chemicals used were of analytical grade and purchased from Qualigens Fine Chemicals (Mumbai, India) and Sigma-Aldrich (Munich, Germany).

Animal Treatment:

Thirty male Wistar rats (80-100 g; 7-8 weeks old) were obtained from the Animal House of the Faculty of Basic Medical Sciences, Ladoke Akintola University of Technology (LAUTECH), Ogbomoso, Nigeria. The animals were allowed to acclimatize for two weeks prior to the experiment while maintained on a standard pellet diet and distilled water *ad libitum*. The rats were weighed and then randomly distributed into five groups of six animals each. Group I was given feed and water only, and served as the Control while Group II received same, in addition to corn oil serving as Oil Control. Group III received 100 mg/l arsenic in the form of sodium arsenite in drinking water. Group IV was administered a homologous mixture of 100 mg DEHP/kg/body weight in corn oil, by gastric intubation. Group V (As + DEHP) received both 100 mg/l arsenic in drinking water and 100 mg DEHP/kg/body weight in corn oil. All treatments were given for thirty days. At the end of the treatment, the rats were fasted overnight and blood was collected into heparinized tubes by cardiac puncture under light diethyl ether anesthesia. The blood was centrifuged at 3,000 *g* for 10 min to obtain plasma. The liver, kidney and brain were removed and homogenized in ice-cold KCl (150 mM). The obtained 10 % homogenate was then centrifuged at 15, 000 g for 15 mins at 4 ^{o}C and the supernatant stored at -20 °C until biochemical analysis.

The doses used for arsenic and DEHP were selected based on previous studies[24,25]. The experiment was carried out in accordance with the LAUTECH Department of Biochemistry guidelines for the care and use of laboratory animals.

Measurement of Ferric Reducing Antioxidant Potential (FRAP) in Plasma and Tissues:

Total antioxidant capacity (TAC) in plasma and rat tissues was determined using the ferric reducing/ antioxidant power (FRAP) assay, developed by Benzie and Strain[26]. Briefly, 1.5 ml of working FRAP reagent (300 mM acetate buffer, pH 3.6, 10 mM 2,4,6-tripyridyl-S-triazine in 40 mMHCl and 20 mM FeCl3 in ratio 10:1:1) pre-warmed at 37 ^{o}C was vortex mixed with 50 mL sample (plasma and in the case of the tissues, 10% homogenate of either liver, kidney or brain). The test was performed at 37 °C and the absorbance was read at 593 nm. Aqueous solutions of known Fe^{2+} concentration were used for calibration.

Determination of Advanced Oxidized Protein Products Concentration:

Spectrophotometric determination of advanced oxidized protein products (AOPP) levels in plasma and tissues was performed by the method described by Witkoet al[27]. Each sample (0.4 mL of plasma, or 10% tissue homogenate) in a tube, was treated with 1.6 ml of Phosphate Buffered Saline (PBS) solution and 0.1 ml 1.16 M potassium iodide (KI). This was followed 2 min later by 0.2 ml of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 2 ml of PBS, 0.1 mL of KI, and 0.2 ml of acetic acid. Concentration of AOPP was calculated by using the extinction coefficient of 26, 000 M^{-1} cm^{-1}.

Measurement of Lipid Hydroperoxides:

The concentrations of lipid hydroperoxides (LOOH) in plasma and tissue homogenates were estimated using the method described by Naurooz-Zadehet al[28]. Plasma or tissue homogenate (90 μl) was mixed with either 10 μl of 10 mMtriphenylphosphine (TPP) in methanol or with 10 μl of methanol and incubated for 30 min at a room temperature. Then, 900 μl of FOX2 reagent (250 μM ammonium ferrous sulfate, 100 μM. xylenol orange, 25 mM H_2SO_4 and 4 mMbutylatedhydroxytoluene in 90 % methanol) was added and the mixture was incubated for another 30 min. The mixture was then centrifuged at 12 000 × g for 10 min to remove flocculated material and the absorbance was read at 560 nm. The absorbance of the sample with TPP was subtracted from the sample without TPP and lipid hydroperoxides concentration was calculated from the standard curve prepared using different concentrations (1–20 μM) of H_2O_2.

Determination of Paraoxonase (PON1) Activity:

Paraoxonase activity of PON1 was determined in plasma and tissue homogenates using paraoxon (O, O – diethyl-o-p-nitrophenylphosphate) as the substrate. The increase in absorbance at 405 nm due to the formation of 4-nitrophenol following the hydrolysis of paraoxon was measured as described by Furlong et al[29]. Sample (20 µL) was added to 760 µL of the assay buffer containing 0.132 mol L-1 Tris-HCl, pH 8.5, and 1.32 mM $CaCl_2$. The substrate, paraoxon in 50 mmol L^{-1}Tris-HCl (200 µl), was added to initiate the reaction. The change in absorbance was continuously monitored on the Genesys 10S UV-VIS spectrophotometer for 3 min. Molar extinction coefficient of 18,050 was used to calculate enzyme activity. One unit of paraoxonase activity was defined as enzyme quantity that disintegrated one micromole of paraoxon substrate in 1 min at 25 °C.

Statistical Analysis:

All data are expressed as mean ± S.D. Data were analyzed by one-way analysis of variance (ANOVA), followed by Tukey test using GraphPad Prism version 5.00 for Windows (GraphPad Software, Inc; CA, USA). Difference at $p<0.05$ was considered statistically significant.

Results:

Exposure to arsenic, DEHP and their combined mixture over a period of 30 days did not yield any apparent signs of toxicity in the rats. The treated animals displayed normal activities as seen with the control animals. No mortality was observed in any of the exposed groups.

Parameters	Control	Oil Control	As	DEHP	As + DEHP
LOOH (μmol L^{-1})	12.33 ± 0.48[a]	14.21 ± 1.02 [a]	21.13 ± 5.62[b]	21.60 ± 0.94[b]	21.60 ± 0.50[b]
AOPP (μmol L^{-1})	114.70 ± 4.53[a]	120.22 ± 3.11[a]	158.30 ± 8.15[b]	150.50 ± 9.65[b]	142.60 ± 4.00[c]
FRAP (μmol mL^{-1})	243.50 ± 13.68[a]	213.47 ± 11.37[a]	116.00 ± 7.00[b]	118.20 ± 10.02[b]	116.30 ± 5.10[b]
PON (U mL^{-1})	176.72 ± 4.74[a]	179.32 ± 5.40[a]	98.56 ± 4.29[b]	86.57 ± 9.17[c]	90.83 ± 5.58[b]

Table 1: Arsenic- and/ or DEHP-induced changes in lipid hydroperoxides (LOOH), advanced oxidized protein products (AOPP), ferric reducing antioxidant power (FRAP) and paraoxonase activity in rats

Values are mean ± SD; n = 6. Values not bearing matching superscript on the same row are significantly different from each other at $P < 0.05$.

Table 1 depicts the effect of exposure to arsenic or DEHP individually or in combination on some selected parameters in the plasma of rats. The plasma TAC was significantly depleted by all three different treatments, with each reducing the relative antioxidant activity in the plasma by more than 50 %. Plasma LOOH concentrations in the rats were significantly increased in all exposures. The observed increases in the groups' plasma LOOH levels were more than 70% of the control groups. The As + DEHP treatment peroxide yield was 57 % and 54 % above arsenic- and DEHP-exposed animals respectively. The coexposure also resulted in a significant increase in AOPP concentration compared to the singly treated groups, although the difference was not statistically significant. Plasma PON activity assayed toward paraoxon was reduced by arsenic, DEHP and As + DEHP exposures. Although the difference among groups was not significant, the reduction in paraoxonase activity was 44 %, 51 % and 47 % respectively, compared to the control animals.

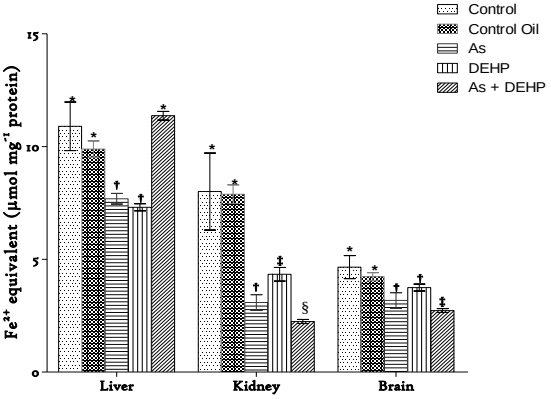

Figure-1: The antioxidant capacity, measured as reducing power with the FRAP, of liver, kidney and brain of rats exposed to arsenic, DEHP and their combined treatment for 30 days. The data are expressed (μmol concentration of equivalent $FeSO_4$) as mean ±SD, n=6 in each group. *, †, ‡, §Differences between values with matching symbol notations within each group are not statistically significant at $P < 0.05$.

All the different treatments induced changes in the antioxidant capacity of the organs, measured as ferric reducing antioxidant power (FRAP). The measured antioxidant capacity revealed differences among the tissues within a group, as well as, among the different treatment groups (Figure 1). The antioxidant capacity in the organs was significantly reduced by the three exposure treatments, except in the liver where concurrent treatment with arsenite and DEHP resulted in significantly higher FRAP value as compared to other treated groups. The coexposure led to 50% and 58% increase in value more than arsenic and DEHP treatment respectively. The FRAP value in the coexposed group, though higher, was not statistically different from the control group. In the kidney and brain of the coexposed group, the antioxidant capacity was significantly lower than either of the single treatments, with the reduction amounting to 27% and 48% in the kidney and 14% and 27% in the brain for arsenic and DEHP treatments respectively.

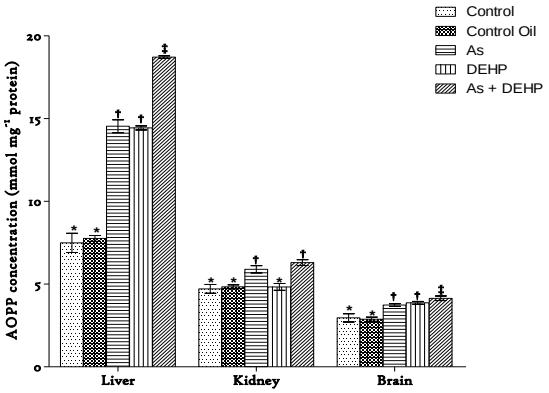

Figure-2: Advanced oxidized protein product (AOPP) concentrations in tissues of rats exposed to arsenite, DEHP and their combined treatment for 30 days. The data are expressed as mean ±SD, n=6 in each group. *, †, ‡Differences between values with matching symbol notations within each group are not statistically significant at $P < 0.05$.

DEHP treatment alone did not affect the advanced oxidized protein products (AOPP) level in the kidney of the rats but the level was significantly increased in all other tissues of the treated groups (Figure 2). In the liver and brain of As + DEHP coexposed rats, AOPP concentrations were significantly elevated more than the levels observed in liver and brain of animals singly treated with arsenicand DEHP. The increases elicited by the coexposure in the liver were 29 % and 30 % above arsenic and DEHP treatment respectively. In the brain, AOPP concentrations were raised by As + DEHP treatment by 10 % and 7 % above arsenic and DEHP treatment respectively.

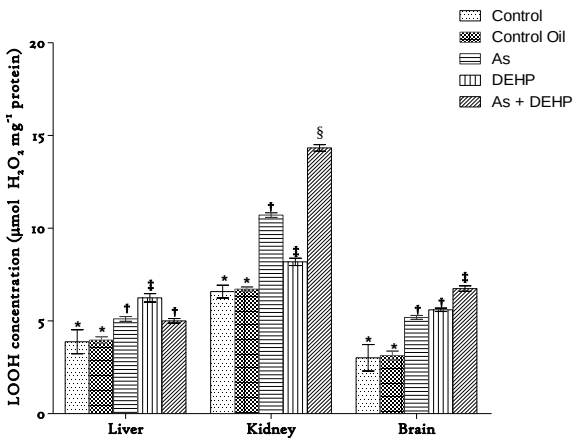

Figure-3: Lipid hydroperoxide (LOOH) concentrations in tissues of rats exposed to arsenite, DEHP and their combined treatment for 30 days. The data are expressed as mean ±SD, n=6 in each group. *, †, ‡, § Differences between values with matching symbol notations within each group are not statistically significant at P < 0.05.

The three treatment regimens led to significantly increased levels of LOOH concentrations in the tissues investigated (Figure 3). In the brain and kidney of As + DEHP group, the treatment resulted in the highest generation of peroxides. As + DEHP treatment caused increases in the brain by 124%, 30% and 21% above that of the control, arsenite and DEHP groups respectively. Similar results were obtained in the kidney with the coexposure generating 34 % and 75 % more peroxides compared to arsenic and DEHP treatment alone respectively.

Significant inhibition of paraoxonase activity was observed in all tissues following treatment with the toxic agents (Figure 4). In the liver, the effect was most pronounced in the As + DEHP group, with 32 % and 48 % inhibition observed compared with arsenic and DEHP exposed groups respectively. In the kidney, the inhibition from the combined treatment was not significantly different from that of DEHP treatment, and both were not as low as what was obtained in the arsenic exposed group. However in the brain, the inhibition of paraoxonase activity in the combined treatment was 57 % and it compared with that induced by arsenic treatment alone.

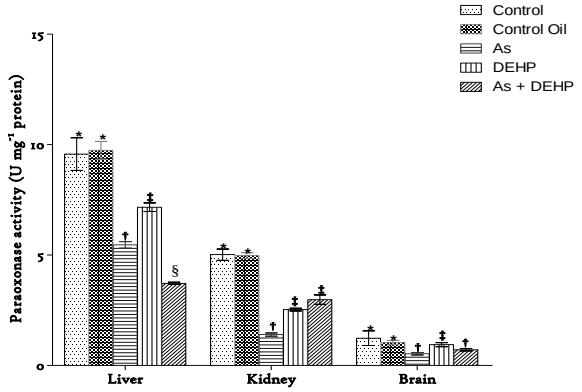

Figure-4: Paraoxonase activity in tissues of rats exposed to arsenite, DEHP and their combined treatment for 30 days. The data are expressed as mean ±SD, n=6 in each group. *,†,‡,§Differences between values with matching symbol notations within each group are not statistically significant at P < 0.05.

Discussion:

Arsenic and phthalates are both ubiquitous environmental contaminants and the adverse effects resulting from exposure to either of them are well documented[6,10]. However, information concerning the consequences of their coexposure is sparse, if not altogether absent in literature. In the present study, the possible deleterious effects of concurrent subchronic exposure to arsenic, in the form of arsenite through drinking water and di(2-ethylhexyl) phthalate (DEHP) by intubation as against individual exposure to these agents were investigated. Biochemical evaluation was made based on oxidative degenerative effects on plasma, liver, kidney and brain of Wistar rats.

Enhanced lipid peroxidation and generation of reactive species are key pathological mediators in many disorders. In our study, combined and individual exposures to arsenic and DEHP significantly increased the levels of lipid hydroperoxides (LOOH) found in the plasma and tissues investigated, an indication of the depletion of the antioxidant scavenger system. These results are consistent with previous reports showing the ability of arsenic and DEHP to induce increased oxidative stress[22,30]. In the plasma, kidney and brain of the treated rats, the increase induced by co-exposure to these agents was statistically significantly more than that observed with either of the individual exposures, suggesting some additive interaction between the two. Interestingly, although arsenite and DEHP elicited significant increases in LOOH levels in the liver, the data on the combined treatment suggest that only arsenite-mediated effect was sustained, with DEHP effect probably inhibited by the presence of arsenite. Similar interaction between toxicants has been reported by Naraharisetti et al, where a high level of malathion inhibited the effect of arsenic in a combined exposure treatment[31].Although the mechanistic basis for our observation in the liver is unclear, the changes could be explained by a stimulation of the liver antioxidant system by the presence of the two agents, which may explain the partial reversal of DEHP effect. This suggestion is collaborated by the increase in liver total antioxidant capacity (TAC) observed in the coexposed group.

The antioxidant capacity of the tissues was determined with the FRAP assay in plasma and organ homogenates. The assay provides a wider picture of the relative antioxidant activity resulting from the individual variations of all antioxidants present in the tissues. Each toxicant significantly reduced plasma TAC by about 50%. Their combined administration did not show a stronger impact on plasma level of TAC than that of the individual agents, showing that there was no appreciable interaction between them. In the liver, although exposure to either arsenite or DEHP resulted in decreased TAC by 29% and 33% respectively, their coexposure interestingly reversed the individual effects, resulting in an increase which was comparable to the control. This suggests that an antagonistic

interaction may exist between the toxicants. Liver, being a source of low molecular weight antioxidants such as reduced glutathione could have been induced into synthesizing more of these antioxidants to counteract the effects elicited by the toxic agents[32]. On the other hand, exposure to arsenite and DEHP singly lowered the total antioxidant capacity by 62% and 46% in the kidney and 32% and 20% in the brain respectively. But of major significance to these organs is the indication of an additive interaction at play in the combined treatment group, with a 72% drop in TAC in the kidney and a 41% reduction in the brain. The toxicants thus, seem to have the ability to enhance each other's effect in the brain and kidney of the rats.

The significant increase in hydroperoxidation as induced by the coexposure of arsenite and DEHP in this study suggests increased generation of free radicals which could cause subsequent oxidative stress-mediated changes in physiological and cellular processes in the affected systems. Arsenicals and phthalates like DEHP have been reported to induce lipid peroxidation and oxidative stress[6,33]. The simultaneous reduction in total antioxidant capacity in the tissues, which is a sign of antioxidant imbalance, indicates that their antioxidant systems were overwhelmed by the interaction of these two agents.

AOPPs are a class of dityrosine-containing protein products formed during (excessive production of oxidants) oxidative stress and accumulate in biological systems, causing damage to biological membranes and endothelium and are considered as novel markers of oxidant-mediated protein damage[27,34]. Their levels are known to reflect the state of generation of free radicals and the degree of protein oxidation. Proteins have many different and unique biological functions, their oxidative modifications can therefore, lead to diverse functional consequences. Our study revealed that arsenite, DEHP and As + DEHP elevated AOPPs in all the tissues investigated except the kidney, where treatment with the phthalate alone seem to have no effect on AOPP concentration. The increase in AOPP observed in the kidney of As + DEHP treated group seemed to have emanated only from arsenic. Our study supported earlier reports that arsenic elevates AOPP and induces protein oxidative damage[2]. The increase in AOPP levels caused by the toxicants singly and in combination indicates an escalation in protein oxidation in the tissues which may result in oxidative injury. Protein oxidation is a covalent modification of proteins as a result of ROS generation. The oxidative modification of target proteins may affect a wide range of cellular functions with severe consequences, since the aggregation of AOPPs are known to be among the pivotal factors underlying a myriad of pathological conditions such as cancer, diabetes, metabolic syndromes and chronic kidney diseases[35,36]. The induced increase in AOPP concentrations observed in the brain, kidney and liver of rats as a result of the coexposure treatment is of great import as emerging evidence have also indicated that AOPPs are not merely a surrogate marker of oxidative stress in injured tissues, but may actually be pathogenic mediators as well[34,37]. Oxidative modification of proteins may involve proteins such as receptors and those implicated in transport systems and signal mechanisms. The accumulation observed may thus, contribute to conditions such as cerebral injury and immunoinflammatory disorder, among others[38,34]. Overall, the increased AOPP levels in the tissues indicated that the animals' ability to protect against oxidative injury had been compromised.

Paraoxonase is found in plasma and tissues such as liver, kidney and brain. It is reported to be an antioxidant enzyme possessing anti-atherogenic and anti-inflammatory properties[39]. In our study, there was significant reduction in the activity of the enzyme, paraoxonase in the plasma and tissues of the rats following exposure to either arsenite or DEHP, as well as, to their combination. The inhibition of the enzyme was observed to be highest in the plasma, liver and brain of arsenite-treated group compared with the DEHP and As + DEHP treated groups. In the liver which is the major site of synthesis of the enzyme[40], arsenite treatment resulted in 46% loss of hepatic activity of the enzyme while DEHP exposure inhibited it by 25%. The combined treatment resulted in a 61% decrease in paraoxonase activity, indicating inhibition of hepatic paraoxonase activity and/or synthesis due to an interaction of arsenite with DEHP. The inactivation of paraoxonase has been reported to be associated with increased oxidative stress and the inhibition may proceed from interaction between the enzyme and oxidized lipids[41]. This has also been observed in our earlier study on cadmium exposure, where reduced paraoxonase activity was accompanied by increased lipid peroxidation[42]. In the present study, the data showing appreciable elevation of LOOH, AOPP and the decrease in TAC induced by the various treatments are indicative of oxidative stress with the consequence of increased oxidative degeneration in the animal tissues. The decreased PON activity in the tissues of the treated rats may, therefore, be a result of increased generation of reactive oxygen species by the toxicants. In addition, increased levels of protein oxidation products have been reported to correlate with low levels of protein SH, the modification of which has been implicated in the inhibition of paraoxonase activity which may be a major factor in the enzyme's inhibition[43,44].

Our study confirmed earlier reports that exposure to either arsenic or DEHP causes oxidative damage in tissues. But more significantly, it demonstrated that their coadministration generally resulted in enhanced oxidative degeneration in the tissues. Though the enhancement of their toxic effects is majorly through an additive interaction, the toxicants were able to display an antagonistic relationship specifically in their effect on hepatic total antioxidant capacity. Further studies however, are required to understand the full

toxicological implications of the changes induced by the co-administration of these environmental agents.

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