Vitamin E Alleviates Diethylhexyl Phthalate-Induced Haematological Changes and Splenic Oxidative Injury in Male Sprague-Dawley Rats

Kazeem A. Akinwumi*

Department of Chemical and Food Sciences, Bells University of Technology, Ota, Nigeria

Abstract: Di-2-ethylhexyl phthalate (DEHP) is a ubiquitous environmental toxicant with high exposure potential to man mainly through leaching from water and foods plastics packages. Exposure to DEHP is associated with cancers, cardiovascular diseases, and splenic toxicity. In the present study, the effect of alpha tocopherol (α- tocopherol) on the DEHP- induced alteration in hematology and oxidative injury in the spleen of male Sprague Dawley rats was investigated. Animals were exposed daily for six weeks to 5 mg/kg bd.wt DEHP and 10 mg/kg bd.wt Vitamin E either alone or in combination. Control rats were given olive oil throughout the duration of the experiment. Organ weight indices and hematology parameters including packed cell volume (PCV), white blood cells (WBC), lymphocytes and neutrophils were determined in test and control rats. In addition, malondialdehyde, superoxide dismustase (SOD), catalase and glutathione-S-transferase (GST) activities were determined in the spleen. Histology of the spleen was also evaluated. Hematoxicity of DHEP was characterized by decreased PCV, WBC counts and neutrophil, while eosinophil was markedly elevated. DHEP administration resulted in oxidative stress that was manifested by elevated MDA and GST coupled with decreases in SOD and catalase activities. The spleen sections from DEHP-treated rats showed congestion and hyperplasia. However, Vitamin E modulated the hematological parameters and alleviated oxidative changes caused by DEHP exposure. Vitamin E also prevented DHEP associated lesions in the spleen. Therefore, Vitamin E could be useful in controlling hematotoxicty and splenic oxidative injury caused by DEHP intoxication.

Keywords: Diethylhexyl phthalate, rat, spleen, oxidative stress, antioxidant, vitamin E.

INTRODUCTION

Diethylhexyl phthalate (DEHP) is a high volume and ubiquitous toxicant that is employed in the manufacture of many industrial and consumer products. Over 2 million tons of DEHP is produced annually, mainly for use as a plasticizer in polyvinyl chloride (PVC) plastics [1]. It constitutes about 40% of the finished PVC plastic by weight [1]. PVC is used in hospital tubing and blood bags, food packages, wires, cable insulation and automobile parts [2, 3]. DEHP founds application in floor tiles, wallpaper, raincoats, toys and auto upholstery [4]. Substantial amount of DEHP is used in the production of fragrance, cosmetics and condenser [5, 6]. Moreover in Taiwan, DEHP is illegally used as a clouding agent in some processed foods including jams, jellies, fruit-flavored juices and beverages as well as in nutraceutical pills and powders [7, 8].

Exposure to DEHP occurs primarily through food [9, 10]. DEHP is loosely bound to PVC and may readily leach into food from packaging materials [11]. DEHP contamination of food can equally occur during processing, storage and transport [9, 10]. Ingestion of DEHP has been shown to occur in bread [12], noddles [13] and meats [14]. Mouthing is also important source of DEHP exposure in infants and toddlers [10]. Hospital patient are exposed to DEHP during blood transfusion and other life-saving protocols involving medical devices [15]. Other sources of DEHP exposure in human include personal care products, medications, house dust, indoor air and soil [16-18]. Plants and animals in the aquatic and terrestrial ecosystems are exposed to DEHP from carelessly discarded plastics and effluent from DEHP related industries [19, 20].

Adverse effects of DEHP exposure include endocrine disruption [21, 22], obesity [23], male infertility [24] and gynecological problems [25]. DEHP exposure is also associated with increased risk of respiratory diseases, attention disorders and adverse reproductive out comes [13, 26-29]. In addition, studies in animals have shown that DEHP is carcinogenic [29]. Furthermore, it exerts potent toxic effects on the different organ in animals including kidney, testes, liver and spleen [5, 30].

The exact mechanism of toxicity of DEHP is not fully established. However, growing body of evidence show that oxidative stress plays a prominent role in DEHP-induced toxicities [22]. *In vitro* and *in vivo* experiments have showed that DEHP activates peroxisome proliferator-activated receptor γ (PPARγ) that results in induction of oxidative stress and disruption of endocrine signaling [22, 31]. The DEHP-induce oxidative stress was characterized by disruption of cellular redox balance in the liver of rats [32].

*Address correspondence to this author at the Department of Chemical and Food Sciences, Bells University of Technology, Ota, Nigeria; Tel: +2348035639816; Fax: 039722620; E-mail: qaakinwumi@yahoo.co.uk

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Therefore, the research into the pharmacological interventions against DEHP-induced toxicities has actively involved modulation with antioxidants including Vitamin E.

Vitamin E (Vit. E) is a lipophilic vitamin with numerous forms. However, α- tocopherol is the most prominent and active out of the eight fat-soluble compounds generally referred to as Vit. E. The α-tocopherol is obtained in diet from several foods including nuts, meat and vegetables. A large population of humans also consumes α -tocopherol as supplements. It is a major component of antioxidant defense system in many tissues. It prevents oxidation of membrane polyunsaturated fatty acids and regulates free radical production and signal transduction [33, 34]. Recently α-tocopherol was shown to protect the organs of male rats subjected to physical excersie from lipid peroxidation [35]. The anti-inflammatory, anti-platelet aggregation and immune enhancement properties of α-tocopherol has also been documented [33]. Owing to these properties, α - tocopherol is believed to protect against many diseases including cancers, cardiovascular, neurological and reproductive disorders [36-39]. Recent studies have reported the antioxidative effect of α - tocopherol against a number of environmental toxicants and drugs including potassium chromate [40], lead [41], cisplatin [42], BPA [43] and cadmium [44]. However, its effect on DEHP toxicity in the spleen is largely unknown.

The spleen is a secondary lymphoid organ that plays vital role in the immune defense and recycling of aging erythrocytes in higher animals. The organ is susceptible to attack by environmental toxicants including DEHP. In a recent study, DEHP damaged the spleen of quail by dysregulating redox balance [5]. In the present study therefore, the role of Vit E in alleviating DEHP-induced alteration in hematological profile and toxicity in the spleen of Sprague Dawley rats was investigated.

2. MATERIALS AND METHODS

2.1. Chemicals

Di-2-ethylhexyl phthalate, 1-chloro-2,4-dinitrobenzene, Reduced glutathione, 2-thiobarbituric acid, hydrogen peroxide H_2O_2 were obtained from Sigma-Aldrich Co, St Louis Mo, USA, while Vitamin E (α – tocopherol acetate) was obtained from Titan Biotech Ltd, Rajasthan, India. Other chemicals used were of analytical grade and procured from standard chemical companies.

2.2. Experimental Animals

Twenty four male Sprague-Dawley rats with the average age of 7 weeks purchased from the Department of Physiology, University of Ibadan, were used for the experiment. The animals were housed and maintained under standard conditions in consonant with the guidelines for use of animals in laboratory experiments [45] and fed with commercially available rat chow (Top Feeds Nigeria Limited) and clean water ad libitum. After one week of acclimatization, the animals were divided into four groups as follows:

Group I – Olive oil

Group II – 5 mg/ kg DEHP

Group III – 10 mg/kg Vit. E

Group IV – DEHP + Vit. E

The selected dose of DHEP is environmentally relevant and is greater than LOAEL for DHEP [46-48]. DEHP and Vit E were dissolved in olive oil and administered orally daily throughout the six week-duration of the study. The final weights of test and control rats were recorded a day after the final treatments and blood was collected by ocular puncture into EDTA tubes for the determination of hematological analysis. The animals were thereafter sacrificed by cervical dislocation. The spleen was harvested, washed in ice-cold KCl, blotted dry on filter paper and weighed.

2.3. Hematological Analysis

The packed cell volume was determined with the conventional microhematocrit method and expressed in percentage as previously described by Lewis *et al*. [49]. The white blood cell (WBC) count for each animal was determined under light microscopy with hemocytometer according to the method of Bjorner M and Zhu [50] using Turk's reagent as diluting fluid. The WBC differentials were determined in blood smears stained with Leishman's stain and examined using an Olympus 41 microscope at 100 × magnification.

2.4. Tissue Processing and Splenic Supernatant Fraction

Each spleen was divided into two and one part was homogenized in 5 times the volume of its weight in cold 0.1M Tris-HCl buffer. The homogenates were centrifuged for 20 minutes at 10000 g and 4°C in a Himac CR21G cold centrifuge. The supernatant was decanted and used for determination of lipid peroxidation and antioxidant enzymes activity.

2.5. Estimation of Lipid Peroxides

Lipid peroxidation in test and control samples was estimated by monitoring the formation of malondialdehyde (MDA) according to the method of Esterbauer and Cheeseman [51]. The MDA concentration was calculated from a molar extinction coefficient $1.56\times10^{5}M^{-1}$ cm^{-1} value [52] and expressed as nmol/g tissues.

2.6. Determination of Cellular Antioxidant Enzymes Activities

Catalase (CAT) activity was determined by monitoring the decomposition of H_2O_2 at 240 nm according to the method of Aebi [53], while super oxide dismutase (SOD) activity was determined at 420 nm based on the competition between the pyrogallol autoxidation by O^{2-} and the dismutation of the radical by SOD as previously described by Marklund and Marklund [54]. Glutathione S-transferase (GST) activity was determined at 340 nm according to the method of Habig *et al*. [55] based on the conjugation of 1-chloro-2, 4-dinitrobenzene.

2.7. Histopathology

The other portion of the spleen from each rat was fixed in freshly prepared 10% buffered formaldehyde (pH 7.0) for 24 h and dehydrated in graded series of ethanol. The tissues were cleared in xylene and embedded in paraffin wax, before 5 µm sections were cut and stained with hematoxylin and eosin on precleaned slides. Histopathological evaluation of the sections was carried out using an Olympus BX 41 microscope at 40X magnification by a trained pathologist, who was blinded to the treatments.

2.8. Statistical Analysis

Data presented as the mean ± SEM were analyzed using the 16th version of Statistical Package for the Social Sciences for Windows (SPSS Inc, Chicago, IL). Multiple comparisons between groups were assessed with one way ANOVA and Ducan Multiple range test. Statistical significant was established when $p < 0.05$.

3. RESULTS

3.1. Spleen Weight and Spleen Weight Ratio

The effect of administration of vitamin E and DEHP on spleen weight and percentage spleen- weight ratio is presented in Figures **1** and **2**. There were decreases in the spleen weight and spleen-weight ratio in the animals given DEHP alone when compared to the control group rats. While the decrease observed in the spleen weight was not significant, that of spleen-weight ratio was significant ($p < 0.05$). The values obtained for both parameters were also similar in the groups given Vit. E alone or with DEHP when both groups were compared with the control.

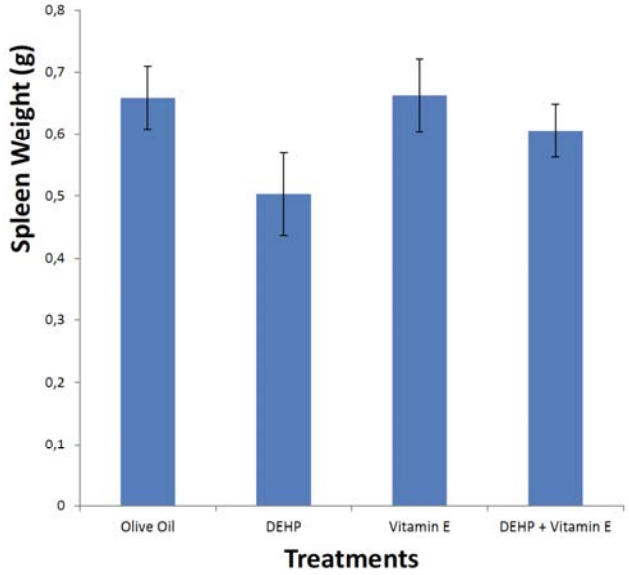

Figure 1: Spleen weight of test and control rats.

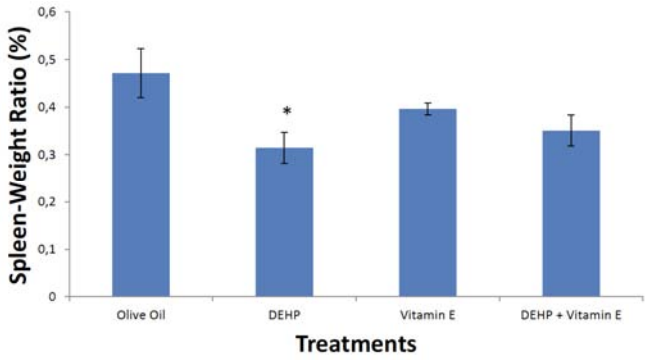

Figure 2: Percentage Spleen-weight ratio in test and control rats.

3.2. Hematology

The effect of administration of vitamin E and DEHP on some hematology parameters in test and control rats is presented in Figures **2-5**. There was a significant ($p < 0.05$) decrease in packed cell volume (PCV) in rats exposed to DEHP when compared to the control. However, PCV values were not significantly different in the groups exposed to Vit. E alone or with DEHP when both were compared with the control. The white blood cell (WBC) count decreased by 23.9% in the group

treated with DEHP when compared to the control. Vit. E marginally improved DEHP-induced decreased in WBC count to 22.1%. When compared to the control, percentage neutrophil in the DEHP-treated group was decreased by 33.3%, while Vitamin E co-administration improved it to 22.1%. DEHP induced marked elevation of eosinophil by 125% when compared to the control, but simultaneous exposure with Vit. E reduced it to 25%. The WBC, neutrophil and eosinophils in the group given Vit E alone were not significantly different from the control. The percentage lymphocytes, macrophage and basophil were similar in all groups (data not shown).

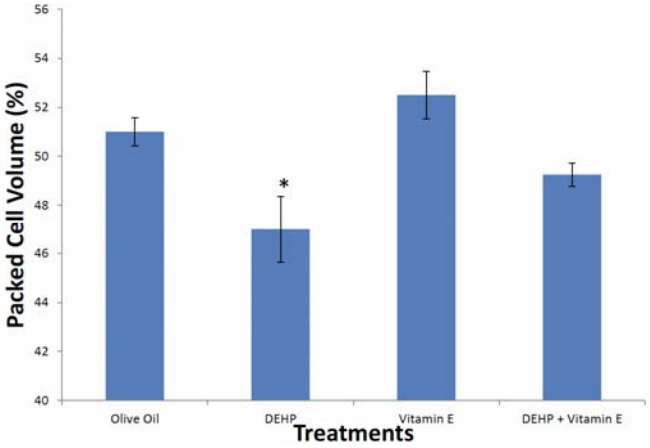

Figure 3: Packed cell volume in test and control rats.

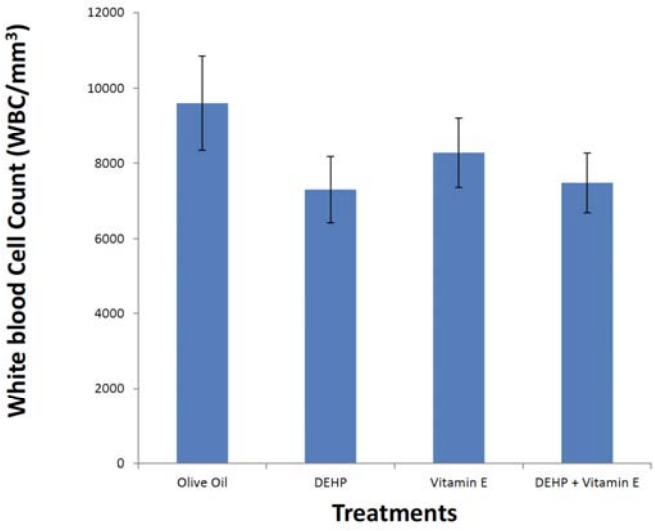

Figure 4: White blood cell (WBC) count in test and control animals.

3.3. Lipid Peroxidation and Antioxidant Enzymes

The effect of administration of vitamin E and DEHP on lipid peroxidation and some antioxidant enzymes in the spleen of test and control group is presented in

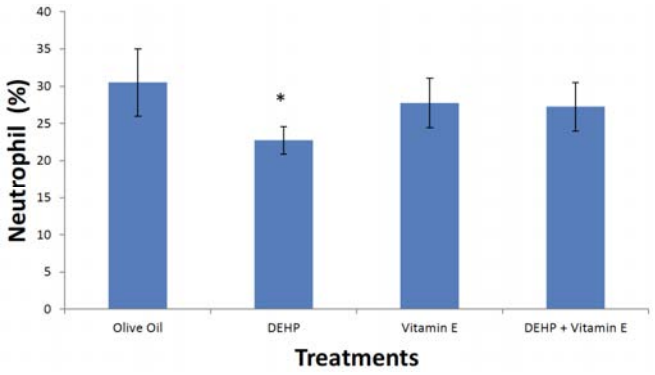

Figure 5: Percentage neutrophils in test and control animals.

Figures **7-10**. The DEHP administration increased malondialdehyde (MDA) level by 95.83 % when compared to the control. However, MDA level was reduced to 75.80% in the group that was simultaneously exposed to Vit.E and DEHP. The MDA levels in group treated with Vit. E alone was insignificantly different from the control group. In the spleen of DEHP-treated rats, catalase activity was significantly ($p < 0.05$) reduced by 36.97% when compared to the control, while concomitant exposure to DEHP and Vitamin E improved it to 15%. Catalase activity was similar in the group that was administered vitamin E only also as compared to the control. A similar trend was observed with SOD activity as DEHP administration resulted in 47.7% decline in SOD activity. In contrast, DEHP-induced reduction in SOD activity was improved to 40% by Vit E, when compared to the control group. The SOD activity in the Vit E only treated rats was not significantly different from the control. The GST activity was however, elevated by 47.6% following DEHP administration when compared with the control. Simultaneous exposure of DEHP and Vit E brought GST activity down to 33.3% also as

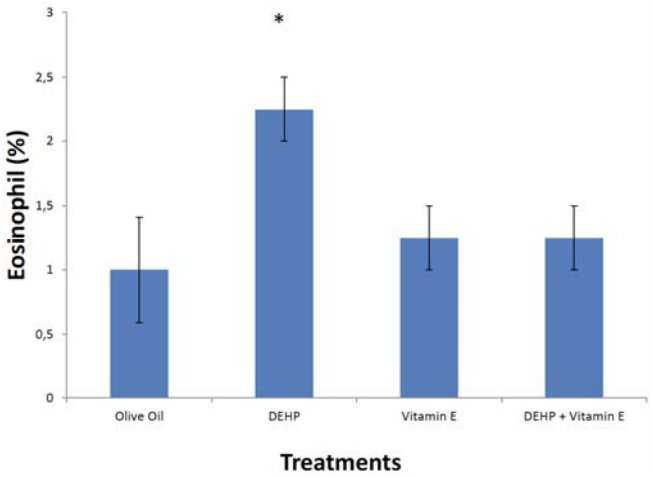

Figure 6: Percentage eosinophil in test and control animals.

compared to the control group. The GST activity in Vit E administered group was similar to the control.

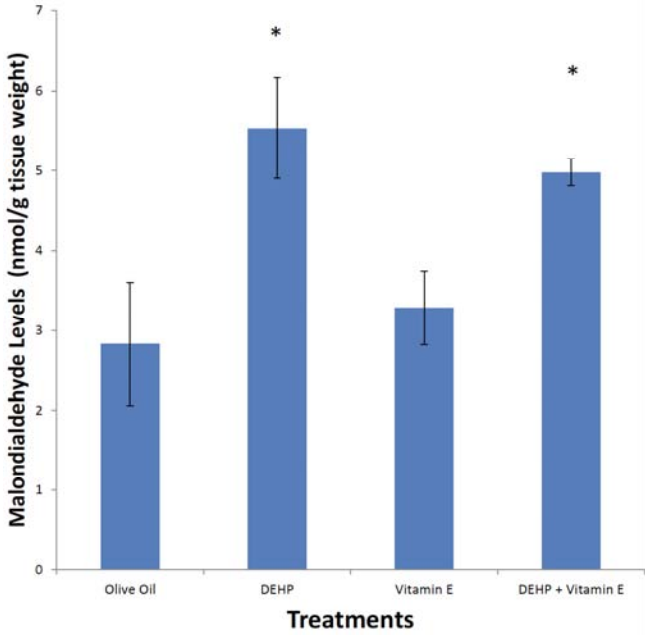

Figure 7: Malondialdehyde levels in test and control animals.

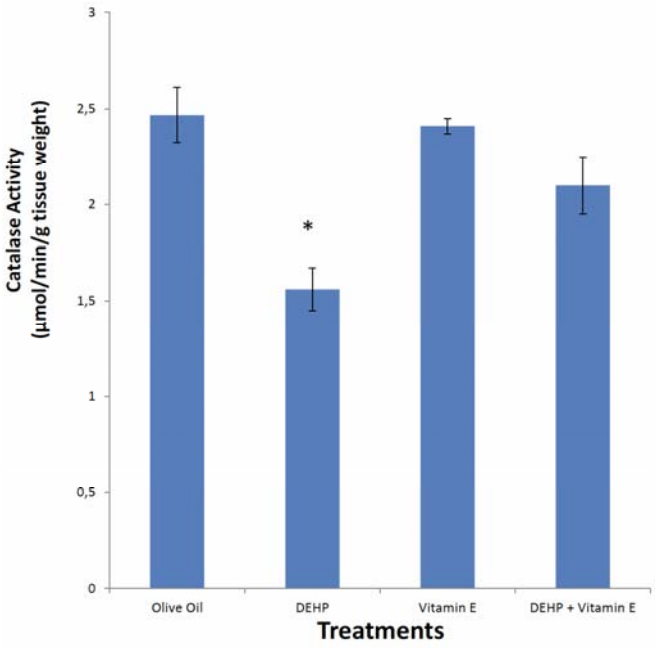

Figure 8: Catalase activity in test and control animals.

3.4. Histological Evaluation

The effect of Vit E and DEHP on the spleen histology of test and control animals is presented in Figure **11**. The spleen of control rats and vitamin E-treated rats showed normal architecture (Figures **11A** & **11D**). The spleen of DEHP-treated rats showed splenic congestion and hyperplasia (Figures **11B** & **11C**). Vitamin E administration to DEHP-treated rats showed normal splenic architecture (Figure **11E**).

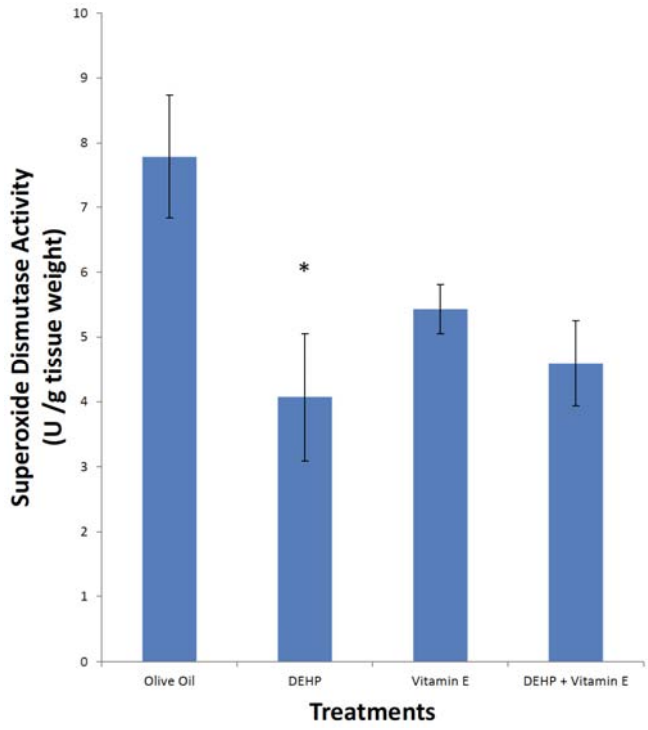

Figure 9: Super oxide dismutase activity in test and control animals.

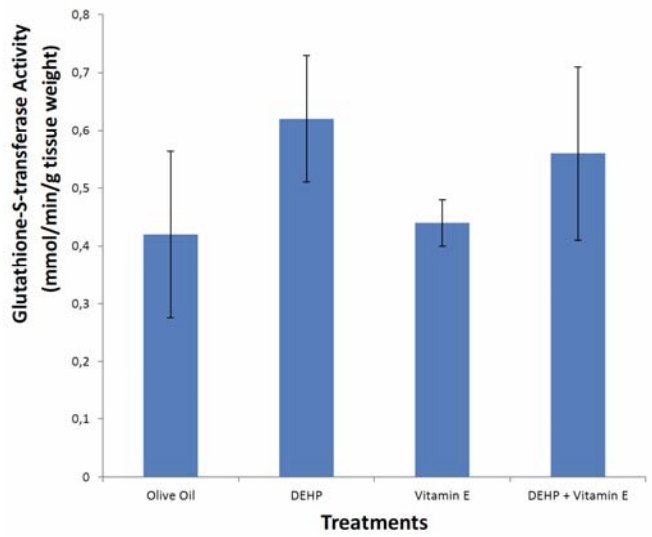

Figure 10: Glutathione -S-transferase activity in test and control animals.

4. DISCUSSION

Dietary antioxidants are vital for maintaining human health. There is accumulating evidence DEHP toxicity is associated with oxidative stress and depletion of antioxidants in several tissue including the spleen [5]. Supplementation with antioxidants therefore could

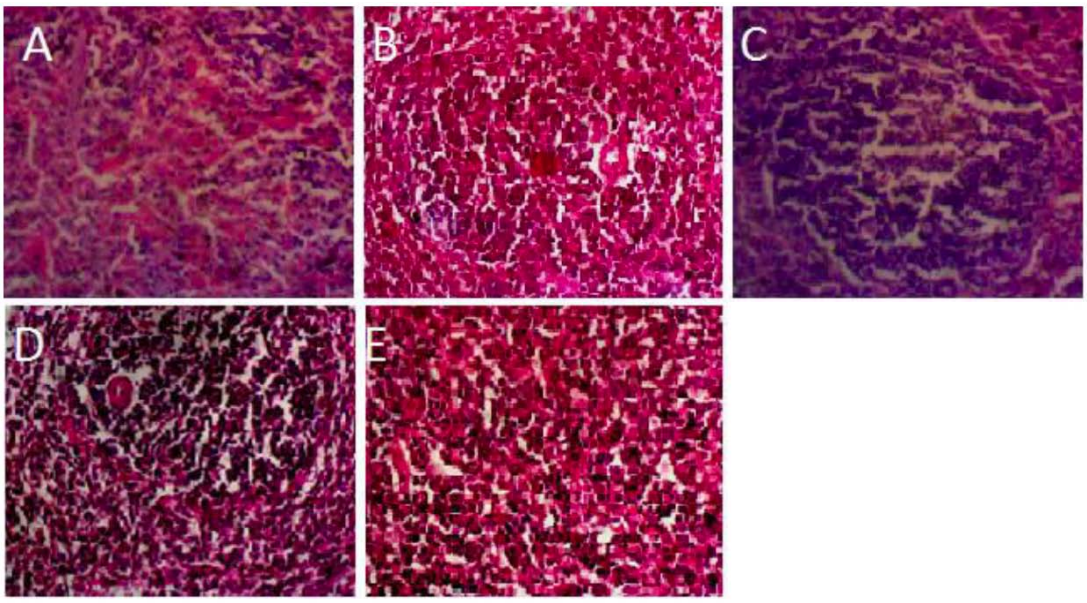

Figure 11: Spleen histology of test and control rats. Control rats with normal histology (**A**), splenic congestion (**B**) and hyperplasia (**C**) observed in DEHP-treated rats, normal spleen histology in rats administered Vitamin E alone (**D**) and normal spleen architecture in rats given vitamin E and DEHP.

retard oxidative injury and adverse effects of DEHP exposure. In the present study, the effect of simultaneous exposure to Vit E and DEHP on blood hematological profile and antioxidative damage in the spleen of Sprague Dawley rats was examined.

The observed decrease in PCV in DEHP-treated rats suggests that DEHP is toxic to erythrocytes and could induced anemia in rats. The data is in agreement with that of Kwack *et al.* [56], who also found a similar decrease in PCV in DEHP exposed rats. Tissue processing of DHEP result in its biotransformation to a more active metabolite, MEHP coupled with the generation of reactive oxygen specie (ROS). Erythrocytes are more vulnerable to ROS-induced oxidation because they are rich in oxygen, iron and polyunsaturated fatty acids that promote lipid peroxidation. In addition, they lack repair mechanism and antioxidant enzyme. Therefore, DEHP-induced erythrocyte damage could contribute to the observed anemia in DEHP-treated rats. Alternatively, ROS can modify proteins including those involved in homeostasis and erythropoiesis, which could result in production of defective erythrocytes that easily undergo hemolysis in circulation. The Vit E supplementation however modulated the decrease PCV caused by DEHP administration by reversing it towards the control value. The protective effect of Vit E on erythrocytes is well documented in human [57-59]. The exact mechanism by which Vit.E protects against erythrocyte damage is unknown. However, it is thought to inhibit premature hemolysis by preventing peroxidation of membrane polyunsaturated fatty acids in erythrocytes [60]. Moreover, it is capable of enhancing erythropoiesis in experimental models and humans [60].

The decline in WBC count observed in the DEHP-administered rats could be an indication of leukopenia. A similar reduction in WBC was reported in male rainbow trout exposed to DEHP [61]. The decrease in WBC suggests that DEHP could inhibit granulopoietic activity in the bone marrow. Moreover, DEHP-induced reduction in total leukocytes count was characterized by low percentage neutrophil and elevated percentage eosinophil in treated rats. Neutrophils are essential component of the immune system that produce cytokines and activate elements of the innate immune response against infectious organisms including bacteria through phagocytosis [62, 63]. They also regulate components of adaptive immune response [64]. Therefore, low percentage neutrophil observed in DEHP-treated rats could be indicative of immunosuppression in treated animals. Increased reactive oxygen species production and subsequent activation of apoptosis in exposed rats may be responsible for the observed neutropenia. In contrast, Vit E boosted leucocytes and neutrophil counts possibly by exerting antioxidant activity against DEHP-induced oxidative stress and apoptosis. Similar improvement in neutropenia that resulted in reduction in the rate of infections was observed in patient with

glycogen storage disease that were administered Vit E [65].

The elevated percentage eosinophil observed in the DEHP- administered is suggestive of eosinophilic response to DEHP and allergic inflammation. The result is in agreement with that of You *et al*. [66] who found a similar increase in eosinophil in the bronchoalveolar lavage fluid of mice exposed to DEHP. The reduction of eosinophil in the co-treated group suggests that Vit E exerts anti-inflammatory action against DEHP-evoked eosinophila.

The spleen plays vital role in the immune defense, erythropoiesis and recycling of aging or damaged erythrocytes. Under stress conditions including leucopenia, cytokines and stress factors are released that may result in contraction of the spleen [67]. Thus, decrease in absolute and relative spleen weight observed in the DEHP group may be due to oxidative stress and splenic toxicity. In the present work, DEHP induced oxidative stress was manifested by elevation of MDA and GST with concomitant decrease in SOD and catalase. This is consistent with previous works in experimental animals treated with DEHP [5, 68]. The free radicals generated during metabolism of DEHP and its intermediates in conjunction with free iron released from recycled erythrocytes in the spleen could enhance the production of the more harmful hydroxyl radicals via Fenton's reaction. Hydroxyl radical could attack membrane lipids resulting in lipid peroxidation in which one of the end products is MDA. High MDA observed in the DEHP-treated animals is therefore, an indication of lipid peroxidation. Lipid peroxidation may modify and inactivate antioxidant enzymes, resulting in accumulation of ROS including superoxide radicals and hydrogen peroxide that are neutralized by SOD and catalase respectively. Inhibition of both enzyme in the present study, suggests that reactive oxygen specie generated in the spleen of DEHP-treated animals overwhelmed their anti-oxidant capacities. Elevation of GST activity may be an adaptive response to the toxicity of DEHP and its metabolites in order to facilitate their elimination from the organ. The elevation of splenic GST activity in the present study is in contrast to that of Yu *et al.* [5], who found decreased GST activity in the spleen of quail exposed to DEHP. The difference in result might be due to variation in specie, doses and physiology of the two organisms. During oxidative stress, tissue may become damaged by excess ROS. This may be responsible for splenic congestion and hyperplasia observed in this study.

Remarkably, Vit E effectively mitigated oxidative stress associated with DEHP exposure in the spleen. The ROS-scavenging and suppressive effects of α- Vit E, was demonstrated in this study by the suppression of lipid peroxidation as evident by the lowering of MDA and modulation of the endogenous antioxidant enzymes. The antioxidant property of Vit E is attributed to the presence of a free hydroxyl moiety on its aromatic ring that can donate hydrogen ion to free radicals leading to the formation a relatively stable tocopheroxyl radicals that can easily be recycled into tocopherol or reduced by glutathione or ascorbic acid [33]. Additionally, Vit.E can also reduce lipid peroxidation rate by preventing the release of Fe^{2+} from their binding proteins [69]. Recently, Wang *et al*. (2017) [70] showed that Vit E reduced DHEP-induced oxidative stress and apoptosis in the testis of rats by reducing PPAR-dependent signaling. The antioxidative effect of Vit E against DEHP damage was confirmed by protection of the spleen against DEHP-induced lesion as evident from the apparently normal histo-architecture in co-exposure group.

CONCLUSION

In conclusion, our data revealed that DEHP exposure can alter blood hematology and disturb redox balance in the spleen of Sprague Dawley rats. Concomitant exposure to Vitamin E alleviates the alterations by attenuating lipid peroxidation and boosting endogenous antioxidant enzyme status. Therefore, Vit E may be useful to counteracting DHEP-associated toxicities.

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