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ORIGINAL ARTICLE

Influence of zinc and gallic acid on haematological alterations, hepatic and intestinal toxicity induced by sub-acute exposure to Dibutyl-*n*-phthalate (DBP) in Wistar rats

Akinleye Stephen Akinrinde[1] · **Asabe Vivian Bello**[1] · **Kehinde Olugboyega Soetan**[1]

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Abstract

Objective Dibutyl-*n*-phthalate (DBP) is utilized industrially as a plasticizer, as well as in consumer products, food processing and medical applications, but there are concerns over its safety. This study investigated the protective effect of Zinc sulphate (Zn) and Gallic acid (GA) against haematological, hepatic and intestinal alterations following sub-acute (14-day) DBP exposure in rats.

Methods Twenty-four male Wistar rats weighing 150–190 g were randomly allocated into 4 groups ($n=6$). Group A (Control) received normal saline at 2 ml/kg. Group B was given DBP (500 mg/kg bw/day) by oral gavage for 14 days. Groups C and D were treated concurrently with Zn (250 mg/kg bw) and GA (120 mg/kg bw), respectively, in addition to DBP treatment.

Results Administration of DBP resulted in significant ($p<0.05$) elevation of serum Alanine transmainase and alkaline phosphatatse, significant ($p<0.05$) increase in faecal counts of coliforms and *Escherichia coli* (Proteobacteria), as well as reduced colonic mucus production and goblet cell numbers. Histological evidence of DBP toxicity included severe congestion of hepatic central venules, severe inflammatory cell infiltration in liver and colonic tissues, and atrophy of colonic mucosal glands, with a reduction in erythrocyte count being the major haematological alteration. The protective effects of Zn and GA were manifested as significant reduction in the activities of serum enzymes and the severity of hepatic and colonic lesions, along with preservation of haematological indices and colonic mucus. GA caused significant reduction in *E. coli* and coliforms, while also increasing enterococci count.

Conclusion Dietary supplementation with Zn or GA may alleviate DBP-induced liver and colonic toxicity. The probable mechanisms may include the preservation of the colonic mucus barrier and improvement in the abundance of beneficial bacteria.

Keywords Zinc · Gallic acid · Phthalate · Faecal microbiota · Mucus · Colon · Liver

Introduction

Phthalates are derivatives of 1, 2-benzendicarboxylic acid (phthalic acid) commonly used as solvents, softeners and additives in a wide variety of industrial and consumer products such as plastics, paints, ink, carpet and cosmetics [1]. Nowadays, the high volume of use of phthalates as plasticizers in manufacturing has greatly increased the tendency for environmental contamination resulting from widespread plastic disposal and leaching of phthalates into water, air and food [2]. Humans and livestock are thus invariably exposed to phthalates when they consume contaminated water, crops and vegetables [3]. Reports have also indicated the illegal use of phthalates as a clouding agent in the production of juices, yoghurt and beverages [4]

Quite significantly, there are growing concerns over the extensive use of low-molecular weight phthalates, such as dibutyl phthalate (DBP) and diethyl phthalate (DEP) as excipients in oral medications, because of their ability to facilitate controlled time release of the active ingredients along the gastrointestinal tract [5]. This potential source of high dose exposure to phthalates is of particular concern to gastroenterologists as the benefits of gastrointestinal

✉ Akinleye Stephen Akinrinde
as.akinrinde@ui.edu.ng

[1] Department of Veterinary Physiology and Biochemistry, Faculty of Veterinary Medicine, University of Ibadan, Ibadan, Nigeria

medications must be weighed along with potential harmful effects of the phthalate excipients. The phthalate, DBP, has been shown to exhibit potentially harmful adverse effects and is found as excipient in gastrointestinal medications and probiotic supplements [6].

Dibutyl-*n*-phthalate is a known to be a developmental and reproductive toxicant, but little is known about its gastrointestinal toxicity [7]. A previous study described the histopathological lesions in the duodenum and jejunum, including decreased villus height and reduced goblet cell numbers in mice treated with 50 and 500 mg/kg doses of DBP [8]. However, only limited evidence is available on the impacts of DBP exposure on critical aspects of mucosal defence including the gut microbiota and mucin secretion. In a recent study by Xiong et al. [9], very low doses of DBP (0.1 and 1 mg/kg) were found to induce significant alterations in the structure and composition of gut microbiota with increases in growth of *Firmicutes* and *Proteobacteria* and reduction in *Bacteroidetes* and *Verrucomicrobia.* Indeed, there is increasing interest in the role of xenobiotic-induced alterations in gut microbiota (gut dysbiosis) as an important contributory mechanism in the causation of many gastrointestinal disorders, as well as disorders of remote organs such as the brain, liver, and heart.

Zinc is the second most abundant trace element in the body after iron [10]. It plays a key role in maintaining all major physiological functions in the body, such as metabolism, signal transduction, cell growth, and differentiation. The beneficial effects of zinc on prevention of gut inflammation has also been demonstrated in mice models and is thought be influenced by the multifaceted roles of metallothioneins, as well as possible contributions by the gut microbiota [11]. Other studies have demonstrated that zinc enhances gut barrier function via its antioxidant activities, while its deficiency has been found to induce damage to the gut membrane barrier [12]. Zinc is known to improve gut microbiota characteristics such as increasing the presence of Gram-negative facultative anaerobic bacteria groups (e.g. *Lactobacilli*), increasing the concentration of short-chain fatty acids (SCFAs) as well as improvement of species richness and diversity [13].

Gallic acid (GA), a naturally occurring polyphenol, is utilized as a nutritional supplement because of its multiple biological activities including antioxidant, anti-inflammatory, antimicrobial and anticancer activities [14]. Several studies support the beneficial role of GA in protection against diseases of the gastrointestinal tract such as gastric cancer [15], colorectal cancer [16] and inflammatory bowel disease [17].

Overall, available data on the impact of DBP on the gut are still very scanty. This study was designed to investigate the protective roles of Zinc sulphate (Zn) and GA on haematology, morphology of hepatic and colonic tissues, as well as alterations in faecal bacterial composition induced by sub-acute exposure to DBP in rats. In addition, this study also examined the response of the mucus layer to DBP exposure and the possible modulation by Zn and GA.

Results

Body weights

The initial body weights were selected such that there were no significant differences in the average weights of the rats (Table 1). At the end of the experiment, the average weight of control rats was significantly ($p < 0.05$) higher than the initial average weight in the same group. Similar increases in body weights of the rats were observed in each of the other groups compared to their initial weights, although the changes recorded were not statistically significant. Furthermore, the average weights of the rats in all the groups were not significantly altered following the different treatments administered.

Haematological effects of Zn and GA treatment on DBP-exposed rats

Haematological evaluation of the experimental rats revealed that rats exposed to DBP (500 mg/kg) exhibited significant reduction ($p < 0.05$) in erythrocyte count with a corresponding increase in mean corpuscular volume (MCV) in comparison with the normal control. Nevertheless, the packed cell volume was not significantly altered across all the groups (Fig. 1a). Administration of Zn at 250 mg/kg and GA at 120 mg/kg significantly improved ($p < 0.05$) the RBC count in comparison with the DBP-exposed rats. Accordingly, rats treated with Zn and GA showed a corresponding lowering of MCV values compared to the DBP-exposed rats. Haemoglobin (Hb) levels in DBP-exposed rats were not significantly different from those of control rats. However, rats co-treated with DBP and Zn exhibited significant reduction ($p < 0.05$) in Hb levels, compared to the normal control and DBP-treated rats, while Hb levels in rats treated with GA

Table 1 Effect of Zn or Gallic acid on body weights of rats exposed to DBP

	Initial body weight (g)	Final body weight (g)
Group A	170.80 ± 10.96	194.40 ± 19.96*
Group B	170.60 ± 13.99	184.20 ± 10.64 ns
Group C	170.60 ± 13.99	183.75 ± 17.52 ns
Group D	171.40 ± 17.62	182.60 ± 19.76 ns

*Indicates significant difference ($p < 0.05$) when compared to the initial weight; ns = not significant. Group A, control; Group B, Dibutyl phthalate, DBP (500 mg/kg) only; Group C, DBP + Zinc sulphate, Zn (250 mg/kg); Group D, DBP + Gallic acid, GA (120 mg/kg)

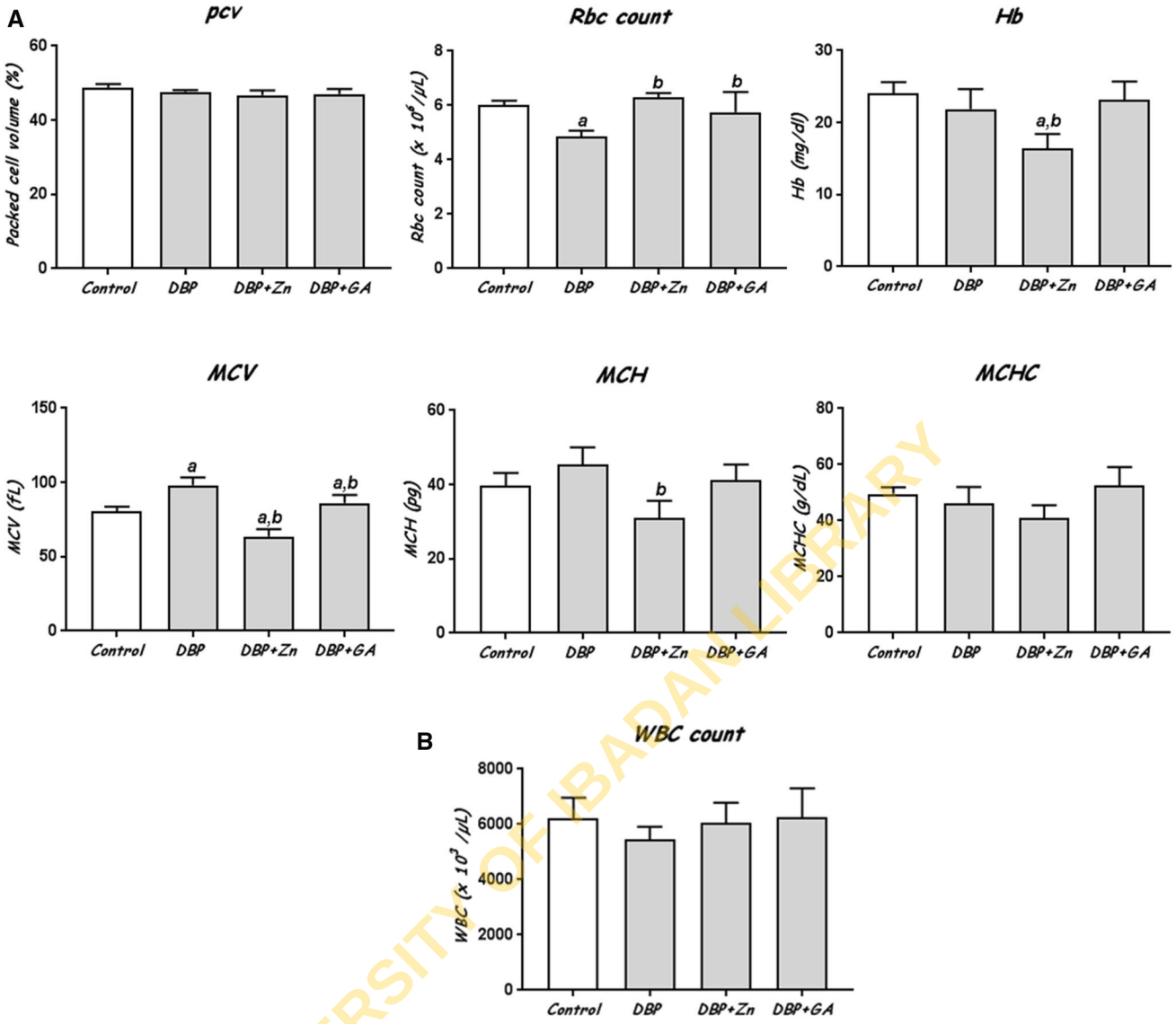

Fig. 1 **a** Erythrocyte indices in Zn- and GA-treated rats during concurrent exposure to DBP. Values are presented as mean ± standard deviation ($n = 5$); Superscript [a] indicates significant ($p < 0.05$) difference when compared with Group A; Superscript [b] indicates significant ($p < 0.05$) difference when compared with Group B. **b** White cell count in Zn- and GA-treated rats during concurrent exposure to DBP. Values are presented as mean ± standard deviation ($n = 5$)

remained unaltered. This lowering of Hb was also reflected in a corresponding reduction in MCH and MCHC values in Zn-treated rats. White blood cell counts (WBC) were not significantly altered in all the experimental groups (Fig. 1b).

Liver function enzymes

The activities of alanine transaminase (ALT), Aspartate transaminase (AST) and alakaline phosphatase (ALP) were determined in the serum to give indication of liver damage. As presented in Table 2, there was significant increase in ALT and ALP activities after DBP administration, compared to control. Administration of Zn (250 mg/kg) significantly attenuated the DBP-induced increases in ALT, AST and ALP, compared to the rats exposed to DBP alone. Similarly, GA (120 mg/kg) administration along with DBP exposure resulted in reduction in the activities of ALT and ALP compared to DBP-exposed rats.

Changes in liver and colon morphology

Liver morphology (H&E)

The morphology of the liver in rats exposed to DBP is presented in Fig. 2. Control rats showed normal liver morphology with normal central venules without congestion,

Table 2 Effects of Zn or Gallic on the activities of liver enzymes in rats exposed to DBP

Parameter	Group A	Group B	Group C	Group D
ALT (U/L)	28.04 ± 1.01	38.45 ± 0.45[a]	24.64 ± 0.76[b]	27.54 ± 0.47[b]
AST (U/L)	68.26 ± 2.46	69.78 ± 0.92	60.00 ± 1.84[b]	66.52 ± 0.44
ALP (U/L)	24.23 ± 3.48	30.82 ± 4.55[a]	24.88 ± 0.81[b]	21.62 ± 1.95[b]

Values are presented as mean ± standard deviation ($n = 5$); Superscript [a] indicates significant ($p < 0.05$) difference when compared with Group A; Superscript [b] indicates significant ($p < 0.05$) difference when compared with Group B. Group A, control; Group B, Dibutyl phthalate, DBP (500 mg/kg) only; Group C, DBP + Zinc sulphate, Zn (250 mg/kg); Group D, DBP + Gallic acid, GA (120 mg/kg)

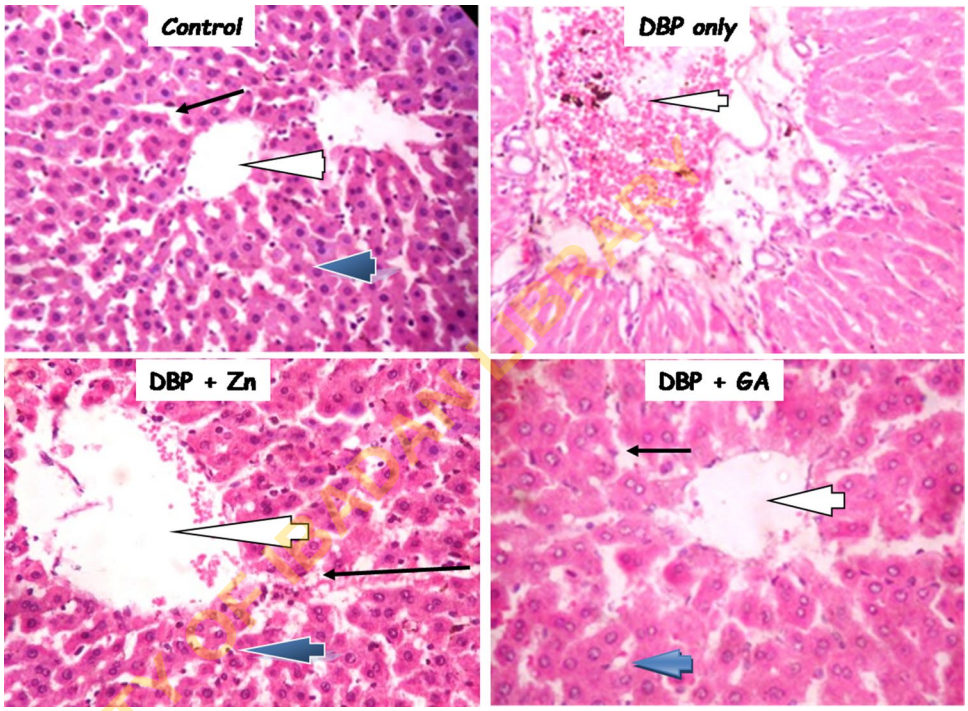

Fig. 2 Morphology of the liver in Zn- and GA-treated rats during concurrent exposure to DBP. Group A (control): hepatocytes show normal morphology; the central venules and sinusoids are devoid of congestion or inflammatory cellular infiltration. Group B (DBP): hepatocytes appear necrotic in some areas; there is severe congestion of the central vein with inflammatory cell infiltration. Group C (DBP + Zn): hepatocyte morphology was well-preserved and the central vein and sinusoids also show normal morphology. Group D (DBP + GA): shows well-preserved morphology of the hepatocytes, central veins and sinusoids

normal hepatocytes and the sinusoids appear normal without inflammatory cell infiltration. In contrast, the liver tissues from rats treated with DBP alone showed central veins with severe congestion and peri-portal infiltration of inflammatory cells (portal triaditis), while the sinusoids also appear mildly infiltrated by inflammatory cells. Treatment of rats with either Zn or GA resulted in obvious preservation of hepatic morphology with the liver tissues showing normal hepatocytes and normal central venules without congestion. The sinusoids also appeared normal and there was no evidence of inflammatory cell infiltration.

Colon morphology (H&E)

The histological analysis of colonic samples revealed lesions indicating damage to the mucosa after DBP treatment for 14 days, compared with control rats given saline treatment (Fig. 3). Notably, rats exposed to DBP alone showed severe inflammatory cellular infiltration, poorly preserved mucosal epithelium and considerably atrophied glands. However, treatment with Zn or GA resulted in attenuation of these lesions, compared to rats exposed to DBP alone. Rats treated with Zn or GA had well preserved mucosal epithelial layer, although mild infiltration of inflammatory cells was still observed in the lamina propria. Nevertheless, the mucosal and submucosal glands were more prominent in these groups than those of the DBP group. Generally, there was no significant change in the colonic muscular layers in all the experimental rats.

Colonic periodic acid schiff staining

Colonic mucus secretion and the integrity of goblet cells were assessed with Periodic Acid Schiff (PAS) as shown in Fig. 4. Treatment of rats with DBP (500 mg/kg) resulted in significant reduction in mucus secretion as indicated by reduced pink-staining PAS-positive regions of the colonic mucosa (Fig. 4a). This was corroborated by a corresponding decrease ($p < 0.05$) in the number of goblet cells per field of colonic sections examined (Fig. 4b).

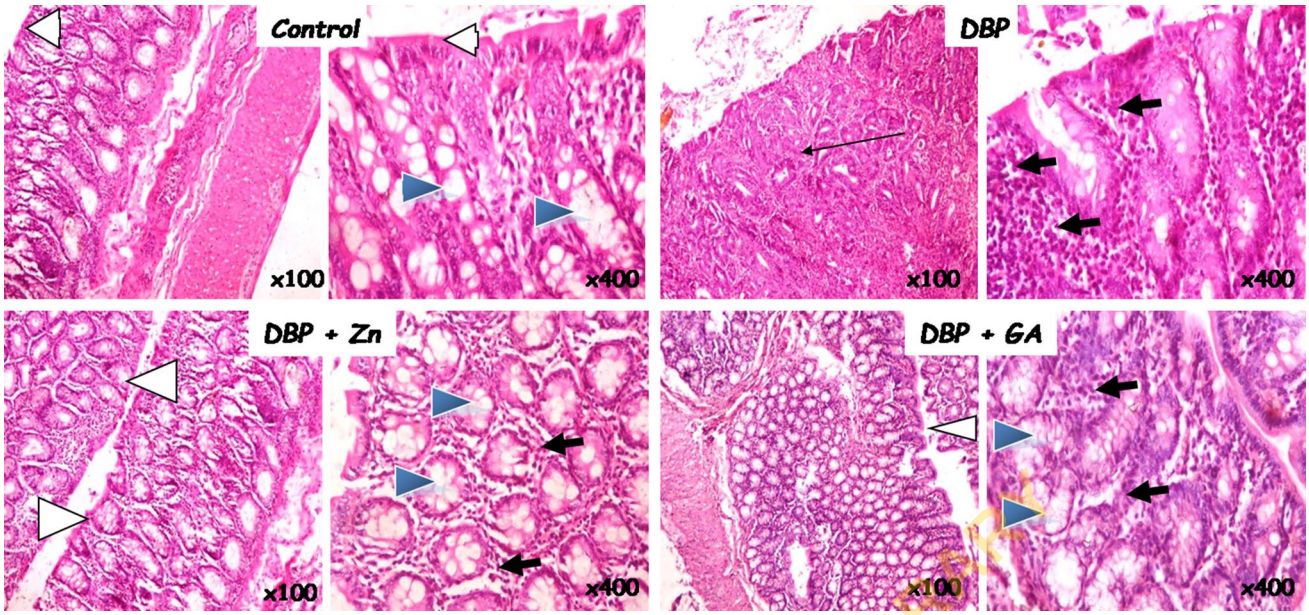

Fig. 3 Morphology of colon mucosa in Zn- and GA-treated rats during concurrent exposure to DBP. Group A (control): shows well-preserved epithelium (*white arrow*) and glands (*blue arrows*) of the mucosa. Group B (DBP): shows poorly preserved epithelial layer with atrophy of glands (*slender arrow*); mucosa is severely infiltrated by inflammatory cells (*black arrows*). Group C (DBP + Zn): there was considerable preservation of the epithelium (*white arrows*) with reduced inflammatory cell infiltration, while glands are also well-preserved. Group D (DBP + GA): glands are prominent and the epithelium is also well preserved; inflammatory cells still noticeable

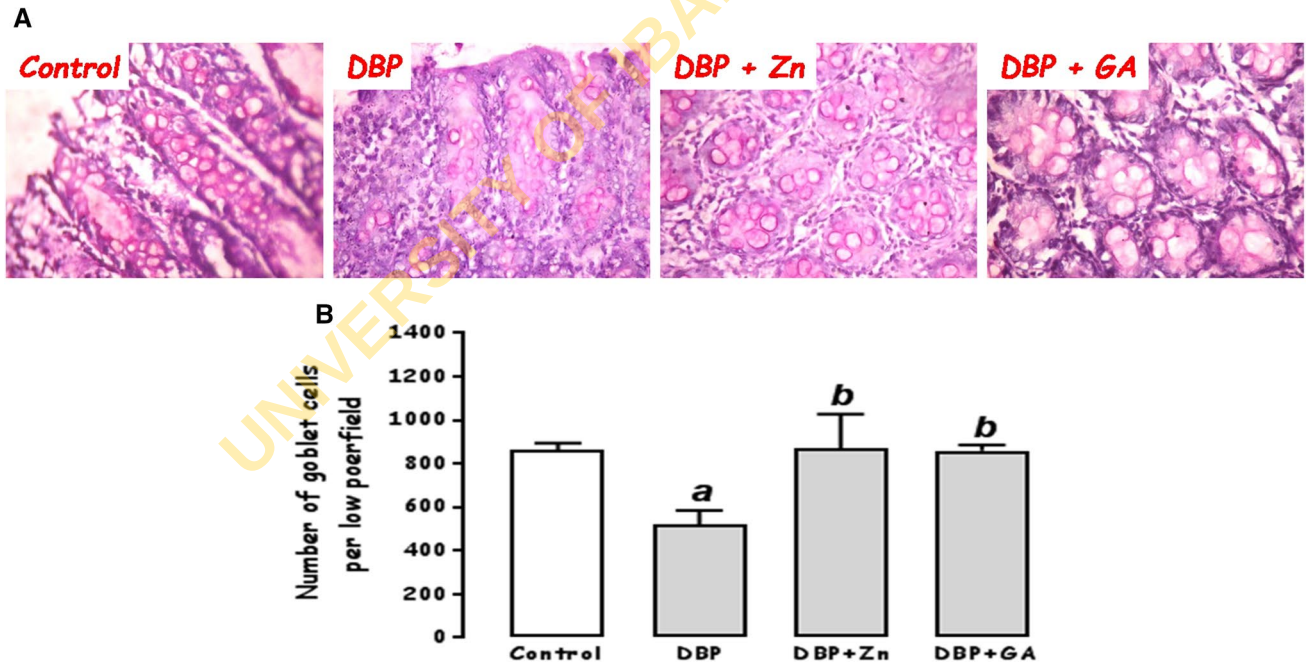

Fig. 4 Goblet-cell count in Zn- and GA-treated rats during concurrent exposure to DBP. **a** PAS staining of the colon of rats after 14 days of exposure to DBP alone and in combination with Zn or Gallic acid. Pink cells are PAS-positive. **b** Quantification of the number of goblet cells per low power field in colon sections ($n = 10$ in each group). Superscript [a] indicates significant ($p < 0.05$) difference when compared with Group A; Superscript [b] indicates significant ($p < 0.05$) difference when compared with Group B

However, rats in the groups co-treated with Zn or GA preservation of the mucus layer with significant increase in PAS-positivity, as well as significant increases in number of goblet cells compared to the DBP group.

Faecal bacteriological analysis

The enumeration on different groups of bacteria in faecal samples of the experimental rats is depicted in Fig. 5. There was a general reduction in total counts of viable bacteria (total heterotrophic count) in faeces from rats exposed to DBP for 14 days compared to the control rats, although the anaerobic population was not significantly affected. However, treatment with Zn or GA restored the balance to control levels. Exposure to DBP caused significant increase in faecal coliform counts, when compared with control, while treatment with GA produced significant ($p < 0.05$) reduction in both coliforms and *Escherichia coli* (Proteobacteria) counts. Enterococci and Lactobacilli (Firmicutes) counts were not significantly altered by DBP administration. However, GA treatment produced significant increase in the growth of enterococci. No growth of *Staphylococci* or *Pseudomonas* was detected.

Discussion

The use of phthalates as excipients in medications used to treat gastrointestinal diseases has justified the need to pay greater attention to their toxic actions, while also developing interventions to mitigate phthalate toxicity in the gastrointestinal tract. In the present study, we have evaluated the influence of Zn or GA supplementation on haematology, liver and colon morphology and function, as well as the alterations of major groups of colonic bacteria in DBP-exposed rats. All rats in the different groups gained weight during the period of the experiment but there were no significant differences in body weight gain between the control group and the treated groups, suggesting that neither feed nor water intake were considerably altered during the experiment.

The most notable haematological finding was a significant lowering of erythrocyte count in DBP-exposed rats, without greatly affecting the packed cell volume. Phthalates such as Dibutyl-*n*-phthalate and butyl benzyl phthalate have been shown to disrupt the redox balance of erythrocytes, causing significant increases in methaemoglobin and reactive oxygen species formation, both of which can lead to an accelerated removal of red blood cells from circulation [18]. This process might have contributed to the lowering of RBC count observed in DBP-treated rats in the present study. In contrast, RBC count was restored to normal values with

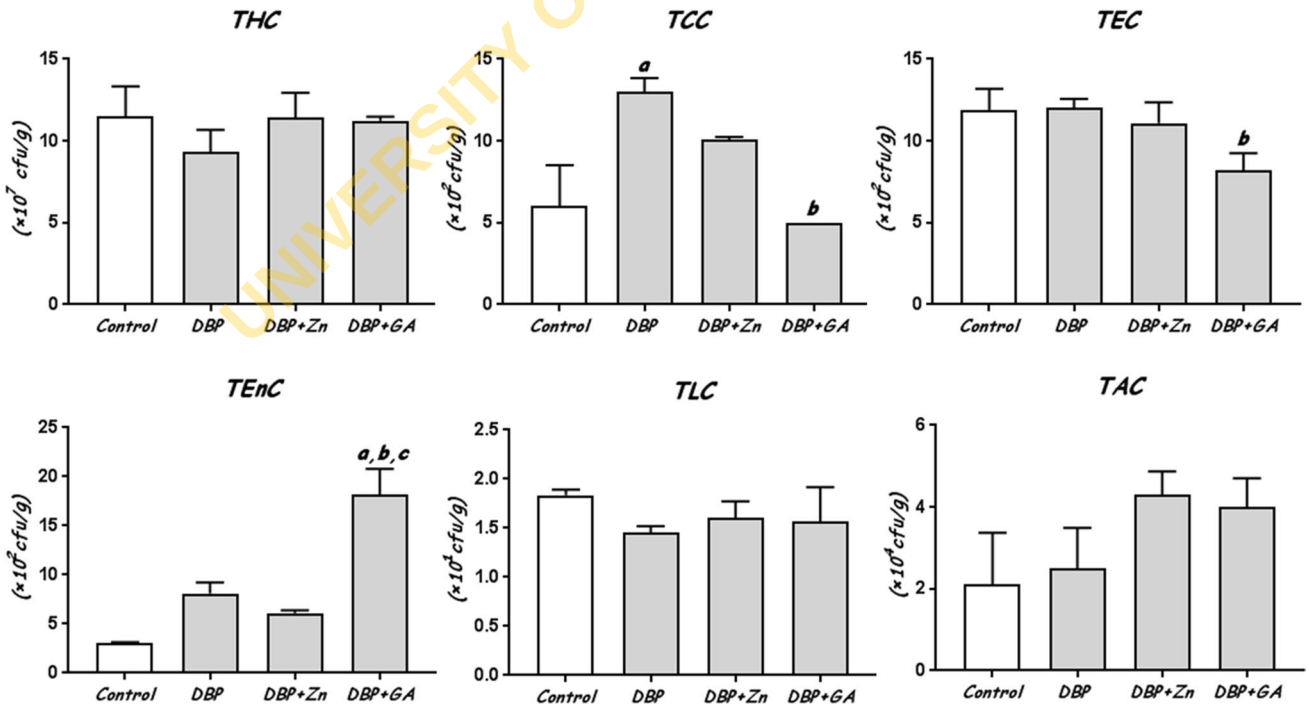

Fig. 5 Faecal microflora in Zn- and Gallic acid-treated rats during concurrent exposure to DBP. THC: Total Heterotrophic Count; TCC: Total Coliform Count; TEC: Total *E. coli* Count; TEnC: Total Enterococcal count; TLC: Total *Lactobacilli* count; TAC: Total Anaerobic Count;

co-treatment with either Zn or GA, suggesting that these compounds could have protected the red blood cells via their antioxidant activities. While Hb levels in DBP-treated rats were lower than that of control rats (percentage decrease: 9.73%), the reduction was not statistically significant.

Interestingly, the rats co-treated with DBP and Zn exhibited significant ($p < 0.05$) reduction in Hb levels, compared to the control and DBP-treated rats, while Hb levels in rats treated with GA remained unaltered. This intriguing finding was thought to be related to interactions that could exist between Zn and iron availability in the synthesis of haemoglobin. It is known that Zn interferes with iron availability by competitively inhibiting the latter's absorption in the intestines. It has indeed been suggested that modest Zn supplementation, while improving Zn indices, tend to induce a cellular iron deficiency [19]. Since iron is an important component of Hb, it follows that Zn supplementation in this study might have interfered with Hb production by restricting iron availability for Hb synthesis. It therefore means that despite reported beneficial effects of zinc on gastrointestinal tissues [11, 20], its supplementation must be done with caution as its interaction with iron during absorption, and possibly during metabolism may affect the status of iron and its associated functions.

The gastrointestinal epithelium is covered by a protective mucus layer composed predominantly of mucin glycoproteins that are synthesized and secreted by goblet cells. Changes in goblet cell functions and in the chemical composition of intestinal mucus are detected in response to a broad range of luminal insults, including chemical exposure [21]. In close association with the intestinal mucus is the gut microbiota which benefits the host by preventing the growth of pathogenic organisms [22] and this relationship has been found to be essential in protecting the host organism from disease conditions such as diarrhoea and colitis, as well as toxic chemicals and drugs such as antibiotics [23]. Following administration of DBP alone at 500 mg/kg for 14 days, there was reduction in colonic mucus secretion which also correlated with decreased goblet cell numbers. Along with this finding, DBP induced a corresponding increase in the growth of coliform bacteria and *E. coli,* which are often classified as pathogenic enteric bacteria. Several pathogenic bacteria are known to have evolved mechanisms that favour disease pathogenesis, including the degradation of mucus glycoproteins, including MUC2, via secretion of bacterial products such as serine proteases that cleave glycoproteins [24]. For instance, enterotoxigenic *E. coli* infection generates an enterotoxigenic *E. coli* autotransporter A (EatA) that degrades mucins [25]. Our findings support the results from a recent investigation in mice where 0.1–1 mg/kg DBP caused decreased mucus secretion and inflammation in the gut [9]. In the same study, DBP reportedly increased the relative abundance of *Firmicutes* and *Proteobacteria*, with simultaneous reduction in *Bacteroidetes* and *Verrucomicrobia.*

In contrast to the effects of administration of DBP alone, concurrent treatment with either Zn or GA resulted in increased mucus secretion and goblet cell numbers compared to the DBP group, showing the possible stimulatory activities of these dietary components on goblet cell activity. Increased mucus secretion has been previously associated with increase in the growth of probiotics such as *Lactobacilli, Enterococcoci* and *Bifidobacteria* [26]. Studies have also shown that probiotics such as *Lactobacilli* induce increase in expression of secreted/gel-forming mucins such as MUC2 and MUC3 which led to a reduction in the adherence of enteropathogenic *Escherichia coli* in the intestinal epithelium [27].

Recent evidence in the in vitro goblet cell model HT-29-MTX indicated that zinc deficiency tends to impair both the amount of mucins secreted as well as the stability of the mucus layer by altering the pattern of glycosylation of mucins [28]. Studies on the effects of GA on colonic mucus secretion are very limited. However, a few studies in vivo and in vitro have suggested that GA can positively influence the growth of gut microbiota in favour of beneficial bacteria, while suppressing the growth of pathogenic ones. At the same time, these studies suggest that intestinal bacteria have the ability to metabolize GA leading to the production of short-chain fatty acids, indicating the possibility of bi-directional interactions between GA and the gut microbiota [17]. In the present study, we found that GA caused significant reduction in potentially pathogenic bacteria, including *E. coli* and coliforms, while the growth of Enterococci was markedly increased with GA administration, compared with the other groups of rats. Additional investigations are required to clarify the mechanisms underlying the ability of GA to modulate gut bacteria, as well as the relationship with mucus production.

Upon histological examination, we found evidence of increased inflammatory responses in both the liver and colonic tissues after DBP administration, which probably supports the increased secretion of mucus in the colon as part of continuous stimulation of the body's immune defences to continued chemical exposure. Rats in the DBP group showed pathological lesions including severe congestion of central venules in the liver with associated inflammatory cell infiltration. The biochemical assays of the activity of liver function enzymes provided confirmation of the histological results with increased activities of ALT and ALP in the DBP group, compared to the control rats. Similar results were obtained by Li et al. [29], who also reported increase in the activities of ALT, AST and ALP in the serum following exposure to DBP at 50, 250 and 500 mg/kg, indicating a compromise of liver function and possible damage to hepatocytes.

Inflammatory lesions were also pronounced in the colonic mucosa of DBP-treated rats, while the degree of inflammation was considerably reduced in the rats treated with either Zn or GA. It was also striking that glands in the colonic mucosa of DBP-exposed rats appeared atrophied, and this further confirms the reduction in mucus secretion in these rats. DBP was previously found to induce inflammation in the lungs via mechanisms involving the inhibition of the Nrf2/TSLP/JAK1 pathway [30], however, there is very little information on the nature and mechanisms of its induction of inflammatory processes in the gastrointestinal tract. Our preliminary findings in this study, therefore, require more in-depth investigation in future studies. The effectiveness of GA in reducing tissue damage following phthalate exposure has also been demonstrated in a recent study by Hosseinzadeh et al. [31], who reported that GA at 50 and 100 mg/kg produced attenuation of Di-(2-ethylhexyl) phthalate (DEHP)-induced oxidative stress and inflammation in mouse testicular tissues.

Conclusion

Administration of DBP to rats led to significant hepatotoxicity and severe inflammatory lesions of the colonic mucosa along with alterations in mucus secretion and the growth of gut microbiota in favour of potentially pathogenic bacteria. Treatment of rats with Zn and GA, on the other hand, resulted in the amelioration of most of the DBP-induced changes, with GA appearing to be more beneficial in this regard. Dietary supplementation with Zn or GA may be considered for the alleviation of DBP-induced liver and colonic toxicity.

Methods

Chemicals, kits and media

Dibutyl-*n*-phthalate was purchased from Loba Chemie Pvt Ltd. (Mumbai, India). Gallic acid (3, 4, 5-trihydroxybenzoic acid) and Zinc Sulphate were procured from Sigma Chemical Co (St Louis, Missouri, USA). The kits for determination of liver enzymes, ALT, AST and ALP were purchased from Randox Laboratories (Ardmore, United Kingdom). MacConkey agar, Eosin Methylene blue agar, Slanetz and Martley medium and Mannitol salt agar were purchased from Oxoid (Basingstoke, United Kingdom); Cetrimide agar (Merck, Germany), De Man Rogosa and Sharpe agar (Himedia, Mumbai, India) were purchased from reputable companies.

Animals and experimental protocol

Male albino rats (Wistar strain), weighing 150–190 g, were procured from the Experimental Animal Unit, Faculty of Veterinary Medicine, University of Ibadan, Nigeria. They were housed in plastic cages in a well ventilated animal house maintained at room temperature of 23 ± 2ºC and a photoperiod cycle of 12 h light/12 h dark. The experimental protocols were in accordance with guidelines outlined in the National Institute of Health publication, "Guide for the Care and Use of Laboratory Animals" [32] and approved by the Institutional Animal Ethics Committee. The rats were initially acclimatized for a week before the commencement of dosing and were fed commercial rat chow and clean tap water ad libitum, throughout the experimental period. Rats were randomly allocated into four groups of six rats each and were treated as follows: Group A served as the control and received normal saline only; Group B were given DBP (500 mg/kg) by oral gavage; Groups C and D were each treated with DBP along with either Zinc sulphate (Zn, 250 mg/kg) or Gallic Acid (GA, 120 mg/kg), respectively. In an initial dosing experiment, rats were subjected to DBP at increasing doses of 10, 100 and 500 mg/kg to assess dose–response effects (data not shown). The dose of DBP used in the present study (i.e. 500 mg/kg) produced the most obvious pathology and was selected in tandem with similar doses used in previous studies [33–35]. The doses of Zn [11, 36] and GA [37] were chosen based on findings reported from previous relevant studies. All treatments lasted for 14 days, at the end of which blood was collected into heparinized and plain sample bottles for the determination of haematological parameters and the evaluation of serum activities of liver transaminases and alkaline phosphatase. The rats were thereafter euthanized by cervical dislocation and the liver and colon were dissected out and placed immediately in 10% phosphate-buffered saline. The tissues were later processed for histopathological examination.

Faecal sample collection and microbial culture

Prior to euthanasia, fresh faecal samples were collected from the different groups and placed in sterile sample bottles in an ice bath, and immediately transferred to the laboratory. All fecal samples were stored at − 80 °C until microbiological analysis using culture methods in selective media as described previously [38, 39]. Briefly, faecal samples were homogenized in tryptone soy broth and 1 ml aliquots were applied on to different selective media including MacConkey agar (Coliforms), Eosin methylene blue (*E. coli*), Slanetz and Bartley medium (*Enterococcus*), De Man Rogosa and Sharpe agar (Lactobacillus), Centrimide agar (*Pseudomonas aeruginosa*) and Mannitol salt agar (*Staphylococcus*).

Bacteria were enumerated as colony forming units per gram of faeces (CFU/g).

Haematological evaluation

Blood samples collected in heparinized bottles were used for the determination of haematological parameters including packed cell volume (PCV) using the microhematocrit method and the haemoglobin (Hb) concentration by the cyanmethemoglobin method. Red blood cell count (RBC) and white blood cell (WBC) counts were determined using the new improved Neubauer hemocytometer. Mean corpuscular volume (MCV), Mean corpuscular haemoglobin (MCH) and Mean corpuscular haemoglobin concentration (MCHC) were calculated from the values of PCV, RBC and Hb using standard formulae. Haematological methods are as described by Jain [40].

Liver function tests

The serum activities of ALT, AST and ALP were determined according to the manufacturer's protocols in the respective kits.

Histopathological analysis

Small pieces of liver and colon tissues were fixed in 10% buffered formalin, dehydrated in graded concentrations of ethanol and then embedded in paraffin wax. Sections of about 5 µm thickness were thereafter made and the tissue slices were stained with haematoxylin and eosin, while some other slides were stained with Periodic acid Schiff, according to standard published methods [41, 42].

Statistical analysis

All quantitative data were expressed as mean ± standard deviation, and differences were determined using analysis of variance (ANOVA) with Tukey's post hoc analysis on GraphPad Prism Software. Significant effect of intervention was noted when $p < 0.05$.

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Author contributions All authors contributed to the conception and design of the study. ASA and AVB handled the data laboratory experiments and data analysis. ASA wrote the first draft of the manuscript. All authors were involved in revising the manuscript.

Declarations

Conflict of interest A. S. Akinrinde, A. V. Bello and K. O. Soetan have declared no conflicts of interests whatsoever.

Ethical statement The experimental protocols were in accordance with guidelines outlined in the National Institute of Health publication, "Guide for the Care and Use of Laboratory Animals" and approved by the Institutional Animal Ethics Committee.

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