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RESEARCH ARTICLE

Aggravative influence of benzene exposure on cyclophosphamide-induced hepatic and myeloid oxidative toxicity in murine model

Olaniyi Solomon Ola [1*], *Ogunwole Iyanuoluwa Aderonke* [2], *Toluwanimi David Ajetunmobi* [2]

[1] Abiola Ajimobi Technical University, Biochemistry and Forensic Science, Ibadan, Oyo State, NIGERIA

[2] Ajayi Crowther University, Department of Chemical Sciences, Biochemistry Unit, Oyo, Oyo State, NIGERIA

ABSTRACT

Cyclophosphamide (CYP) is a potent alkylating chemotherapy with broad clinical utility in cancer treatment. Benzene (BZ) is a ubiquitous solvent which poses threat to human health as it causes hematological disorder and liver damage. This study was designed to evaluate the influence of BZ co-exposure on rats undergoing CYP treatment with special consideration to some biomarkers associated with liver and blood tissue. Twenty-four male Wistar rats were divided into four groups (n = 6) and were orally treated with CYP or exposed intranasally to BZ individually or in combination for 28 days. The results revealed that exposure to either BZ or CYP caused significant increase in the plasma transaminases, alkaline phosphatase and bilirubin content related to control group. Significant decreases in hepatic superoxide dismutase, catalase and reduced glutathione concentrations were also observed. Similarly, exposure to either the compound affected the biomolecules by causing the significant increase in the plasma Advanced oxidized protein products and hepatic malondialdehyde concentration. The exposure to either BZ or CYP also caused an alteration in hematological parameters and significantly increased the pro-inflammatory markers: myeloperoxidase enzyme, NF-κB and TNF-α relative to control. The combined exposure to BZ and CYP demonstrated the enhanced toxic effect inferred from the examined biomarkers when compared with their individual treatments. In conclusion, this study therefore proves the enhanced toxicity of CYP chemotherapy when animals are co-exposed to BZ in the environment. It may therefore be recommended that individuals undergoing CYP treatment should avoid exposure to BZ to guide against the enhanced side effect of this anticancer drug.

Keywords: cyclophosphamide, hepatotoxicity, benzene, inflammation, toxicity

Correspondence:

Olaniyi Solomon Ola

Address: Biochemistry and Forensic Science, Abiola Ajimobi Technical University, Ibadan, Oyo State, NIGERIA

Email: olaolaniyis@yahoo.com

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INTRODUCTION

Periodic exposure to various hazardous environmental molecules can influence the potency or toxicity of therapeutic drugs [1, 2]. Individuals are exposed to countless number of both environmental and exogenous compounds in lifetime that are absorbed through our diet, water and air and may therefore impact human diseases [3]. Co-exposure to environmental toxicants with drug molecules may impact drug efficacy. Studies on the aflatoxin B1 and various medicinal plant, for example, have shown drug-exposure interactions [4]. The menace of toxicity of anticancer agents raised a major global concern. The off-target toxicity of many drugs generally determines their level of clinical importance and acceptability. The scientific knowledge on mixture effects can play major role in the advancement and development of methods to assess risks from combined exposures to both drugs and toxic xenobiotics [5, 6]. The toxicology profile of drug most often only includes underneath health conditions and probably co-drugs in combination therapy and previous drugs used. The emergence of toxicological response due to mixture effects even when all individual chemicals are present at no-effect concentration has been demonstrated in a broad range of complex chemical mixtures [7, 8]. The literature on

inclusions of environmental factors that individuals are exposed to in their various occupational environment while under drug regimen is lacking. However, influence of benzene (BZ) exposure in environment in relation to chemotherapeutic agents have not been systematically investigated to date.

BZ is a ubiquitous chemical found in industrial workplaces, car exhaust, and myriads of everyday products [9]. This aromatic hydrocarbon is a reputable leukemogenic substance that also damages blood-forming tissues [10]. During its metabolism, it is transformed in liver by cytochrome P450 to produce byproducts like phenol and benzoquinone that are toxic to bone marrow and other organs [11]. Research has shown that BZ can make other chemicals more dangerous by ramping up certain enzymes, depleting the natural antioxidants and interfering with cellular repair systems [12].

Cyclophosphamide (CYP) is an important immunosuppressant and alkylating chemotherapeutic agent employed extensively in oncology [13, 14]. This drug requires hepatic bioactivation through cytochrome P450 enzymes primarily CYP2B6, CYP2C9, and CYP3A4, to generate 4-hydroxycyclophosphamide [15]. CYP therapy causes injuries to normal tissue [16] and peroxidative damage to liver which are linked to generation of reactive oxygen species (ROS) [17]. The metabolic activation of CYP by the liver cytochrome-P450 enzymes generates metabolites such as acrolein and phosphoramide. Phosphoramide metabolite offers anticancer and immunosuppressive action while acrolein interferes with the antioxidant defense mechanism of the body by inducing the production of ROS [18, 19].

The examination of metabolic pathways of both BZ and CYP may suggest potential interaction between them. Both BZ and CYP are metabolized in the liver by cytochrome enzymes, especially CYP2E1 and CYP2B6 [20]. Moreover, the two substances affect the hematopoiesis processes [21, 22]. The shared metabolic pathways raise the possibility of interactions that could influence the toxicity of CYP when patient undertaking the treatment is simultaneously exposed to BZ in the environment. Understanding how BZ exposure might affect the toxicity of CYP is crucial for safety of patients, especially for those undergoing treatment with alkylating chemotherapy who may also be exposed to BZ at work or in environment. This study therefore investigates the potential effect of BZ exposure on the rats undergoing CYP treatment focusing on liver and blood parameters. More importantly, our findings could help medical practitioners and health workers in disseminating medical advices to patients who might encounter BZ exposure while receiving CYP treatment.

MATERIALS AND METHODS

Reagents and Chemicals

5,5-dithiobis-2-nitrobenzoic acid (DTNB), glutathione (GSH), 1-chloro-2,4-dinitrobenzene (CDNB), hydrogen peroxide (H_2O_2) and epinephrine were all purchased from Sigma Chemical Company (London, UK). CYP tablet is got from West Coast Pharmaceutical Works Ltd, Gota, Ahmedabad, India. Kits for alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and total Bilirubin were obtained from Randox laboratories ltd. (Antrim, UK). All other reagents were of analytical grade obtained from British drug house Poole England.

Experimental Animals

Twenty-four male Wistar rats weighing 170-200 g were obtained from the animal facility and acclimatized to laboratory conditions for one week prior to the experiment. The animals were housed in standard plastic cages under controlled environmental conditions. All animals had free access to standard laboratory chow and water ad libitum throughout the experimental period. The rats were randomly divided into four experimental groups of six animals each. Group A (control) served as the negative control and received only standard laboratory feed and water. Group B (BZ alone) received intranasally administered BZ at a dose of 400mg/kg body weight daily for 4 weeks. Group C (BZ + CYP) received combined treatment consisting of BZ at 400mg/kg body weight intranasally and CYP at 2mg/kg body weight intraperitoneally and daily for 4 weeks. Group D (CYP alone) received intraperitoneal administration of CYP at a dose of 2 mg/kg body weight daily for 4 weeks.

Blood and Liver Sample Collection

Twenty-four hours after the last treatment, each rat received an intraperitoneal injection of 0.04% colchicine two hours before sacrifice. The blood samples were immediately collected from the retro-orbital plexus and the animals were then sacrificed by cervical dislocation. The liver was carefully excised from each animal. The liver tissues were processed to evaluate both enzymatic and non-enzymatic antioxidant systems, while lipid peroxidation was assessed through malondialdehyde (MDA) measurement.

Assessing Liver Function and Cholestasis Markers

Activities of liver biomarker enzymes and bilirubin in plasma were measured using established spectrophotometric methods. AST and ALT activities were determined following the procedure described by Reitman and Frankel [23]. This method involves the formation of hydrazone compounds with 2,4 dinitrophenylhydrazine, monitored at 546 nm using a spectrophotometer. ALP activity was assessed based on the principle developed in [24]. The methodology using dimethyl sulphoxide developed in [25] was used for bilirubin estimation.

Table 1. Influence of BZ exposure on plasma activities of ALT, AST, ALP, and bilirubin level in CYP-treated rats

Groups	AST (U/L)	ALT (U/L)	ALP (nm/min/ml/mg/protein)	BILIRUBIN (µmol/L)
CONTROL	31.48 ± 3.83	20.00 ± 2.65	43.30 ± 2.75	3.15 ± 0.99
BZ ALONE	40.22 ± 3.55* (27.76%)	27.98 ± 2.25* (39.90%)	59.15 ± 5.19* (36.61%)	3.95 ± 2.00# (25.39%)
BZ + CYP	43.84 ± 1.93# (39.26%)	31.73 ± 3.40* (58.65%)	80.13 ± 13.48# (85.06%)	4.87 ± 0.67* (54.60%)
CYP ALONE	37.98 ± 3.48* (20.65%)	26.88 ± 2.12# (34.40%)	62.05 ± 34.45* (43.30%)	4.10 ± 2.10 (30.15%)

Note. Data are expressed as mean ± standard deviation for six rats in each group.
* Significantly different from the control ($p < 0.05$)
# Significantly different from cyclophosphamide group

Analysis of Antioxidant Molecules and Enzyme Activities in the Liver

Reduced GSH concentration was determined following the method of [26], which employs a colorimetric reaction with 5,5′-dithiobis-(2 nitrobenzoic acid) that produces measurable absorbance at 412 nm. SOD activity was assessed using the technique described in [27]. Catalase activity was evaluated according to Singha method [28].

Plasma and Liver Protein Content Determination

Protein concentrations in both plasma and liver homogenate were quantified using the Biuret method as described in [29].

Evaluation of Oxidative Stress Markers in Plasma

Plasma advanced oxidation protein products (AOPPs) were measured using a modified approach based on the method described in [30]. The procedure involved mixing 100 µl of plasma with 400 µl of phosphate buffer saline solution. Subsequently, 25 µl of 1.16 M potassium iodide was added to the mixture, followed by 50 µl of acetic acid after a 2-minute interval. The absorbance was immediately measured at 340 nm using a spectrophotometer, with readings taken against a blank solution containing 500 µl of PBS, 25 µl of 1.16 M potassium iodide, and 50 µl of acetic acid.

Assessment of Oxidative Lipid Damage in the Liver

MDA levels, serving as an indicator of lipid peroxidation, were quantified in liver tissue using the thiobarbituric acid (TBA) assay described in [31]. Liver homogenates react with TBA under acidic conditions, forming a colored complex with maximum absorbance at 532 nm. The absorbance was measured spectrophotometrically, and MDA concentrations were determined by comparing the readings to a standard curve. Results were expressed as micromoles per milligram of liver tissue.

Assessment of Pro-Inflammatory Cytokines and Enzymes in Inflammation

The inflammatory response was evaluated by measuring the levels of TNF-alpha, NF-kB, and myeloperoxidase (MPO) activity in liver tissue using ELISA and spectrophotometric techniques. TNF-alpha and NF-kB concentrations were determined using sandwich-ELISA kits, where optical density values at 450nm were directly proportional to the cytokine concentrations present in the samples. MPO activity was measured spectrophotometrically using O-dianisidine and hydrogen peroxide as substrates, with optical density readings taken at 470 nm. These assays were conducted following the manufacturers' protocols and the methodology outlined in [32].

Assessment of Hematological Parameters

Blood samples collected in ethylenediaminetetraacetate bottles were used for hematological analysis. The parameters evaluated included hemoglobin (HGB) concentration, hematocrit percentage, mean corpuscular volume, mean corpuscular hemoglobin, red blood cell (RBC) count, white blood cell (WBC) count, lymphocyte count, monocyte count, granulocyte count, and platelet (PLT) count. All measurements were performed using standard automated blood analyzer.

RESULTS

Influence of BZ Exposure on Plasma Activities of ALT, AST, ALP and Bilirubin Level in CYP-Treated Rats

The results from **Table 1** demonstrate that individually BZ and CYP significantly elevated liver enzymes AST, ALT, and ALP compared to control groups, indicating liver damage. Most notably, the combined exposure to BZ and CYP (BZ + CYP group) produced the most severe hepatotoxic effects, with AST activity reaching 43.84 ± 1.93 U/L, ALT activity at 31.73 ± 3.40 U/L, and ALP activity at 80.13 ± 13.48 U/L, suggesting a synergistic toxic interaction between these compounds.

Effect of BZ Exposure on Hepatic Activities of Superoxide Dismutase and Catalase in CYP-Treated Wistar Rats

Figure 1 shows that exposure to BZ significantly reduced hepatic SOD and catalase activities in exposed animals compared to control animals, indicating a compromised antioxidant defense system. CYP treatment similarly caused significant decrease in SOD and catalase enzymes, demonstrating drug-induced oxidative stress. The combined BZ and CYP group exhibited the most severe reduction in both SOD and catalase activities, suggesting synergistic toxicity. These findings confirm that BZ pre-exposure

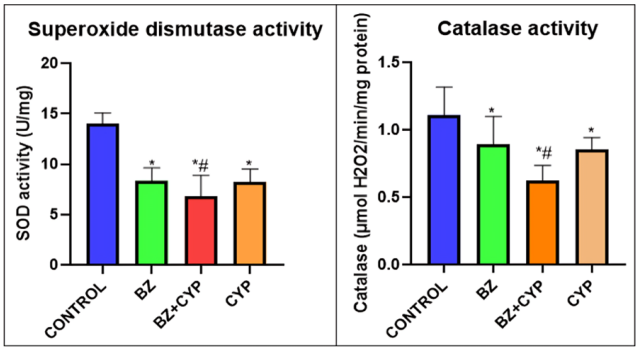

Figure 1. Influence of BZ exposure on hepatic activities of superoxide dismutase and catalase in CYP-treated Wistar rats (Data are expressed as mean ± standard deviation for six rats in each group; *Significantly different from the control ($p < 0.05$); & #Significantly different from cyclophosphamide group)

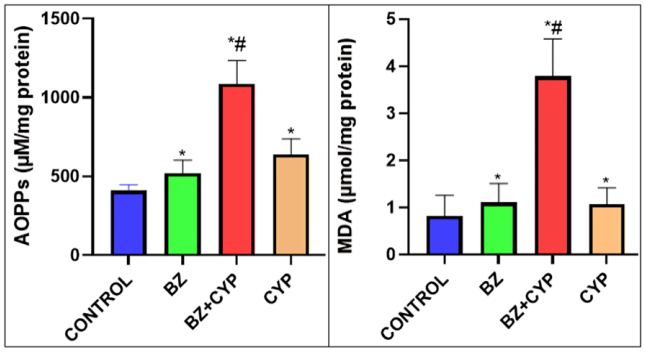

Figure 2. Influence of BZ on plasma concentration of advanced AOPP and MDA in CYP-treated Wistar rats (Data are expressed as mean ± standard deviation for six rats in each group; *Significantly different from the control ($p < 0.05$); & #Significantly different from cyclophosphamide group)

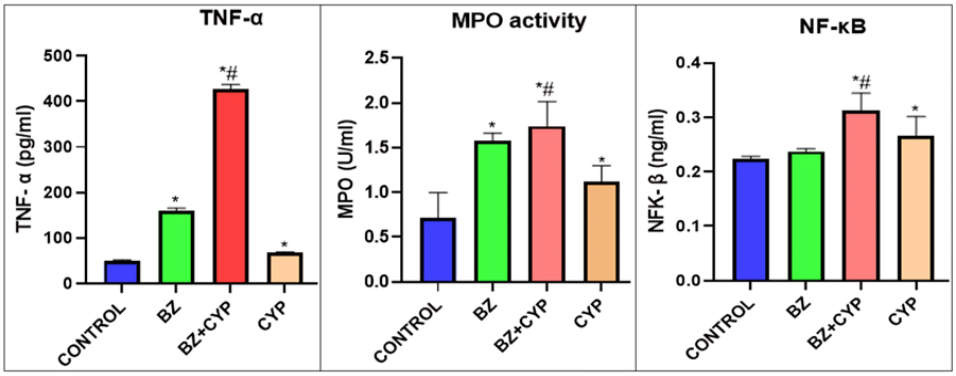

Figure 3. Influence of BZ exposure on pro-inflammatory cytokines and enzymes in CYP-induced hepatotoxicity in Wistar rats (Data are expressed as mean ± standard deviation for six rats in each group; *Significantly different from the control ($p < 0.05$); & #Significantly different from cyclophosphamide group)

potentiates CYP-induced hepatotoxicity by further depleting the liver antioxidant capacity.

Effects of BZ on Plasma Concentration of Advanced Oxidation Protein Products and MDA in CYP-Induced Hepatotoxicity in Wistar Rats

Figure 2 illustrates that BZ exposure significantly enhances CYP-induced oxidative stress in Wistar rats, as indicated by significantly increased levels of AOPPs and MDA in the combined treatment group as compared to the CYP-only group.

Effects of BZ Exposure on Pro-Inflammatory Cytokines and Enzymes in Inflammation in CYP-Induced Hepatotoxicity in Wistar Rats

Figure 3 shows that BZ exposure significantly worsened CYP toxicity in Wistar rats through inflammatory mechanism. BZ and CYP independently caused significant increase in the levels of TNF-α, MPO activity, and NF-κB, but the combined exposure causes a surge elevation in TNF-α, MPO activity, and altered NF-κB levels beyond either treatment alone. The combined treatment of BZ and CYP produced more severe toxic effects than individual treatments, indicating that BZ exposure makes the body more sensitive to CYP damage through enhanced inflammation and cellular stress responses.

Influence of BZ Exposure on Hematological Parameters in CYP-Treated Rats

This study examined the hematotoxic effect of BZ exposure on blood parameters in CYP-treated rats. The results in **Table 2** reveal that both BZ and CYP significantly reduced WBC counts independently compared to controls, with BZ exposure decreasing WBC from $7.43 \pm 3.83 \times 10^3/\mu L$ to $3.96 \pm 0.49 \times 10^3/\mu L$ and CYP reducing it to $4.02 \pm 0.79 \times 10^3/\mu L$. More importantly, the combined exposure group (BZ + CYP) demonstrated the most severe myelosuppressive effects, with WBC count dropping to $2.75 \pm 0.42 \times 10^3/\mu L$ from $7.43 \pm 1.32 \times 10^3/\mu L$, indicating a synergistic hematotoxic interaction that could significantly compromise immune function.

DISCUSSION

Human exposure to BZ through long-term inhalation in contaminated working environment and its attendant health implication has been widely reported. It is also known that

Table 2. Influence of BZ exposure on hematological parameters of CYP-treated rats

Group	WBC (10 × 9/L)	Lym (10 × 9/L)	Gran (10 × 9/L)	RBC (10 × 12/L)	HGB (g/dL)	HCT (%)	PLT (10 × 3/uL)
CONTROL	7.43 ± 1.32	6.41 ± 1.43	0.47 ± 0.73	7.93 ± 0.35	16.24 ± 0.38	46.50 ± 1.12	733.00 ± 228.02
BZ ALONE	3.96 ± 0.49	3.39 ± 0.80	0.07 ± 0.04	7.54 ± 0.34	15.90 ± 1.17	43.62 ± 2.53	810.00± 146.30
BZ + CYP	2.75 ± 0.42	2.63 ± 0.38	0.05 ± 0.02	7.29 ± 0.57	13.04 ± 0.37	42.22 ± 1.11	912.2 ± 136.52
CYP ALONE	4.02 ± 0.79	3.55 ± 0.77	0.29 ± 0.38	7.68 ± 0.32	14.90 ± 1.05	44.24 ± 2.11	912.6 ± 11.746

Note. Values are mean (X) ± standard deviation; Data are expressed as mean ± standard deviation for six rats in each group.
* Significantly different from the control ($p < 0.05$)
# Significantly different from cyclophosphamide group

BZ interact with other substances, toluene and xylenes and thereby potentiates its effects or vice versa [33].

Contamination of the environment with BZ has been related to factors that predispose individuals to development of some health issues such as cancers, liver diseases and onco-hematologic abnormalities [34]. Exposure of humans to BZ is still far from elimination since it is commonly used solvent and many industries find its use very inevitable [35]. CYP is a commonly used chemotherapy drug that has shown effectiveness in treating various cancers and autoimmune diseases. Moreover, CYP usage is relevantly sustained because of its potent chemotherapeutic effect. There is need to determine potential interaction between BZ and CYP because of their metabolic pathways. Both BZ and CYP are metabolized in the liver by cytochrome enzymes, especially CYP2E1 and CYP2B6 [20]. This shared metabolic pathway raises the possibility of interactions that could influence the toxicity of CYP or vice versa when patient undertaking the treatment is simultaneously exposed to BZ in the environment. Therefore, this study aims to investigate the hypothesis that exposure to BZ might influence the toxicity of CYP during treatment regimen, focusing on liver and blood parameters.

The hematological system is particularly vulnerable to cytotoxic effects because of the rapid turnover of blood cells and the high metabolic activity of bone marrow [36]. WBC are essential components of the immune system whereas RBC and HGB are crucial for oxygen transport while PLTs are necessary for blood clotting [37]. Myelosuppression, characterized by decreased production of blood cells, is a common toxic effect of both chemotherapy drugs and environmental toxins [38]. In this study BZ and CYP individually altered hematological parameters leading to reduction in WBC, RBC, HGB and packed cell volume. The observed reduction in WBC could weaken the immune system and heighten the risk of infections [39]. The decrease in WBC count in CYP group corresponds to the information on its myelosuppressive effects as a chemotherapy drug [40].

Co-exposure to BZ and CYP resulted in further reduction of WBC count to 2.75 ± 0.42 x 10^9/L, indicating a potentially severe hematotoxic effect.

AST, ALT, and ALP are essential enzymes used to assess hepatocellular damage [40]. AST and ALT are transaminases that leak from damaged liver cells into the bloodstream, while ALP indicates cholestasis or bile duct dysfunction [41]. Bilirubin is a byproduct of HGB breakdown which accumulates when liver function is impaired [42]. In the present study, BZ and CYP individually induced significantly higher activities of ALT, AST, ALP and bilirubin level (left part in **Figure 1**) compared with the control. This finding supports earlier research indicating that BZ and CYP can individually lead to liver toxicity and affect liver enzyme activities [43, 44]. More interestingly, co-exposure to both BZ and CYP increased the activities of the enzymes relative to either BZ or CYP alone suggesting enhanced toxicity induced by BZ synergy. This increased liver toxicity when both compounds are present suggests that simultaneous exposure could pose additional risks to patients undergoing CYP therapy.

The study further examines key markers of oxidative stress to understand the mechanisms behind the heightened toxicity starting with antioxidant enzyme activities. Superoxide dismutase is a vital antioxidant enzyme that protects cells from oxidative stress by converting superoxide radicals into hydrogen peroxide and molecular oxygen [45]. SOD activity is often measured as a marker of antioxidant defense capacity [46, 47]. Catalase is also an important antioxidant enzyme that protects cells from oxidative damage by breaking down hydrogen peroxide [48]. Catalase enzyme works in conjunction with SOD to maintain cellular redox balance and prevent oxidative damage to cellular components. The study showed significant ($p < 0.05$) declined changes in SOD and CAT activities across the BZ and CYP treatment group relative to control, illustrating the oxidative burden caused by BZ and chemotherapy drug. The decrease in SOD and catalase activities due to BZ and CYP exposure aligns with studies reporting harmful effects of these substances on various antioxidant enzymes by weaken antioxidant defenses in the liver [49, 50]. Importantly, the combined administration of BZ and CYP further decreased catalase and SOD activities in liver cells compared to the CYP group ($p < 0.05$). This suggests that BZ exposure may worsen the liver toxicity induced by CYP, possibly by impairing the antioxidant defense system.

MDA is one of the end products of lipid peroxidation that serves as a reliable indicator of oxidative damage to cellular

membranes [51, 52]. When cellular antioxidant defenses are overwhelmed, ROS attack polyunsaturated fatty acids in cell membranes, leading to lipid peroxidation [53]. The reduction of antioxidant enzymes leads to increased oxidative damage which can be assessed through MDA levels [54]. The results showed that CYP administration significantly raised MDA levels in liver cells compared to the control group. Moreover, co-exposure of rat under CYP treatment to BZ further increased MDA levels compared to the CYP alone group. The additional increase in MDA levels with BZ co-administration indicates a synergistic effect between CYP and BZ in promoting lipid peroxidation and resultant oxidative stress in the liver.

AOPPs are markers of oxidative stress that form when plasma proteins react with chlorinated oxidants [55]. AOPPs formation represents a more advanced stage of oxidative damage, where proteins undergo structural modifications that can impair their function and contribute to cellular dysfunction [56]. Results showed that CYP administration significantly raised AOPPs level in liver cells compared to the control group. Furthermore, co-exposure of rats taking CYP to BZ further raised AOPP levels compared to the CYP-only group. The increase in AOPP levels after CYP administration suggests that this drug induces oxidative stress in the liver, potentially contributing to liver toxicity.

NF-κB is a key transcription factor involved in different cellular processes, including inflammation, immune response, and cell survival [57]. NF-κB remains inactive in the cytoplasm by associating it with its inhibitory kappa B subunit. It is activated through multiple stimuli especially oxidative stress which induces NF-κB phosphorylation and degradation of inhibitory kappa B that causes translocation of NF-κB to the nucleus where it regulates the expression of numerous genes involved in inflammation, immune responses, and cell survival [58, 59]. Genes that encode cytokines especially TNF-α are regulated by NF-κB [60]. Therefore, the activation of NF-κB in turn leads to the production of pro-inflammatory cytokine, tumor necrosis factor-alpha (TNF-α) [61]. The oxidative stress demonstrated through antioxidant enzyme depletion and lipid peroxidation activates inflammatory pathways through NF-κB and TNF-α [61]. The results indicated that CYP administration significantly raised NF-κB and TNF-α levels in liver cells compared to the control group. Additionally, CYP treated rats that were co-exposed to BZ showed further increase in NF-κB and TNF-α levels compared to the CYP-only group. The increase in NF-κB and TNF-α amounts after CYP treatment suggests that this drug activates inflammatory responses in the liver, due to liver toxicity [62]. The further rise in inflammatory markers with BZ co-administration indicates a synergistic effect between CYP and BZ in promoting inflammation and cellular stress in the liver. This finding aligns with earlier studies that showed that BZ is a strong inducer of oxidative stress and inflammation [63].

MPO is an enzyme primarily found in neutrophils and is released during neutrophil activation and degranulation [64]. MPO catalyzes the formation of hypochlorous acid from hydrogen peroxide and chloride ions, which serves as a potent antimicrobial agent. However, elevated MPO levels may create a cycle of inflammation and oxidative stress [65]. MPO-derived oxidants can activate NF-κB, leading to more pro-inflammatory cytokines that recruit additional neutrophils to the site of inflammation [66]. The result shows that CYP administration significantly raised MPO levels in liver cells compared to the control group. Co-exposure to BZ together with CYP treatment further increased MPO activity compared to the CYP-only group. The enhanced inflammation may result in chronic liver damage that can increase the risk of developing more serious liver issues. This self-perpetuating cycle shows how initial oxidative stress can escalate into chronic inflammatory liver damage.

In conclusion, the present study showed that BZ and CYP exhibited significant toxicity on liver and blood during individual exposure whereas there is a definite synergistic trend of simultaneous exposure to BZ and CYP, that is, an enhanced toxicity as compared to the individual effect of each substance. It can therefore be suggested that exposure to BZ during CYP treatment may enhance myelotoxic and hepatotoxic effects of CYP in rats.

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AI statement: No generative AI or AI-based tools were used in this study.

Declaration of interest: No conflict of interest is declared by authors.

Data sharing statement: Data supporting the findings and conclusions are available upon request from the corresponding author.

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