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Dose-Dependent Impact of Ascorbic Acid on Bone marrow Cellularity Against benzene-induced pre-leukemia in Rats

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KEYWORDS

Benzene-induced pre-leukemia, Ascorbic Acid, Bone-marrow cellularity, Blast cell

ABSTRACT:

Background: Benzene is known to lead to bone marrow failure, oxidative DNA damage (micronucleus formation), pancytopenia, and pre-leukemic changes that can develop into acute myeloid leukemia (AML). Although no specific protective agent is available, vitamin C (ascorbic acid) may attenuate oxidative stress and augment hematopoiesis.

Objective: To assess graded doses of vitamin C versus the benzene-induced effects on the bone marrow and pre-leukemic state in rats.

Materials and Methods: Rats were divided into 6 groups: control, benzene-only or benzene plus vitamin C (100, 200, 500, or 1000 mg/kg/day, oral) for 4 weeks. Pre-leukemia was induced by administration of benzene intravenously (0.2 mL of 1:5:5 benzene:propanol:distilled water) every 48 h; endpoints of bone marrow micronucleus indices (%PCE, %NCE, MN-PCE, MN-NCE, PCE/NCE), CBC with differential, platelet status, inflammatory ratios (e.g., NEU/LYM, SII), and the percent of peripheral blood blast. Results: Benzene induced severe genotoxicity (↓%PCE and PCE/NCE; ↑%NCE, MN-PCE, MN-NCE; $p<0.0001$), leukopenia (WBC ↓37%) with lymphopenia and monocytopenia, neutrophilia, basophilia, eosinophilia, platelet abnormalities and 35% circulating blasts (0% in control). Vitamin C conferred dose-dependent protection by increasing the number of markers in the marrow, correcting WBC and differential balance, enhancing platelet count/morphology (MPV, PDW), returning SII elevation to normal, and reducing blasts to 3-4% at 1000 mg/kg.

Conclusions: Vitamin C (100-1000 mg/kg) provides potent, dose-modulated protection of anti-hematotoxic effects and genotoxic properties against benzene, with the highest dose reaching control values and inhibiting pre-leukemic development.

1.0 Introduction

Acute myeloid leukemia (AML) is a fatal, aggressive hematologic malignancy resulting from clonal proliferation of immature myeloid cells in the bone marrow, leading to bone marrow failure and high mortality. It is primarily a disease of adults with a poor prognosis despite available treatment, which has generated interest in novel preventative and therapeutic strategies (Testa et al., 2021). Benzene exposure is a well-established risk factor for AML among known leukemogenic factors. Benzene is a common, industrial chemical and environmental pollutant with known myelotoxicity and may induce aplastic anemia, myelodysplastic syndromes (pre-leukemic conditions), and acute leukemia in humans and in animal models (Döhner et al., 2022; Elazab et al., 2022). Its blood-system toxicity results from reactive metabolites (e.g., benzoquinones) that stimulate oxidative stress, DNA damage, and chromosomal abnormalities in hematopoietic stem and progenitor cells. Consequently, exposure can cause pancytopenia (global decrease of blood cell counts) and bone-marrow dysplasia with elevated immature blasts, which can eventually progress to overt leukemia (Yusoff et al., 2023). An important characteristic of benzene genotoxicity is the production of micronuclei in bone marrow erythroid cells, mirroring chromosomal damage or loss. A bone marrow micronucleus assay of MN-PCE (as well as of the PCE/NCE ratio) is widely utilized to detect such DNA damage in vivo, as well as to evaluate bone marrow proliferation. It has been reported that exposure to benzene caused the measurement of micronucleus

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frequencies to increase a good deal in rodent bone marrow and peripheral blood due to genomic instability due to leukemic transformation (Hasan Khudhair et al., 2022). Because of the ubiquity and deadly blood-related effects of benzene, many researchers have been interested in agents able to reduce benzene-induced toxicity and the leukemia-associated risk associated with exposure. So far, no single chemopreventive agent is sufficiently effective to protect against benzene hematotoxicity (Rehan et al., 2023). However, a variety of natural products with antioxidant and anti-inflammatory effects are being studied for the protection potentials. Specifically, in rats with bovine lactoferrin iron-binding glycoprotein possessing antioxidant activity, benzene-induced hematotoxicity was significantly mitigated, consequently, normal cellularity of bone marrow and peripheral blood counts were restored. Additionally, in experimental models, dietary antioxidants, including polyphenols, decreased benzene-induced cytogenetic injury and hematologic effects. These data, collectively, support the theory that fortified endogenous antioxidant defenses can fight benzene's oxidative and genotoxic stress (Zeghib et al., 2021). Vitamin C, or ascorbic acid, is one of the candidates, as its antioxidant and cytoprotective profile is known to be quite good. Vitamin C is a water-soluble essential vitamin that neutralizes reactive oxygen species and helps regenerate other antioxidants within the body. Furthermore, it is also associated with immunological maintenance and inflammation control. It has been demonstrated that vitamin C supplementation is beneficial in the restoration of redox status within pathologic states, minimizing oxidative injury and lipid peroxidation (Kumar & Rizvi, 2023). In a recently published animal model, vitamin C shielded the tissue from drug-induced organ toxicity, and helped the patients to mitigate the damage incurred by these agents (Azzam et al., 2025). Furthermore, high-dose vitamin C has recently drawn interest in oncology for its possible anticancer potential. At pharmacological concentrations (achievable via intravenous infusion), ascorbate could be a pro-oxidant against the tumor microenvironment, releasing hydrogen peroxide and selectively inducing apoptosis in tumor cells (Sun et al., 2025). In particular, in vitro and early clinical studies have shown that high-dose ascorbate can have potent leukemic cell apoptotic effects and potentiate standard therapeutic agents (Testa et al., 2021). Vitamin C, for example, induced apoptosis in acute lymphoblastic leukemia cells by accentuating endoplasmic reticulum stress following in vivo administration (Sun et al., 2025). Simultaneously, vitamin C might enhance anti-tumor immunity in immune responses; it is reported to synergize with immune checkpoint inhibitors and other immunotherapies, which can render tumor progression less responsive (Yin, 2024; Kim et al., 2025). These multifaceted anti-tumor activities underscore vitamin C's therapeutic potential in malignancies including leukemias.

In addition to its direct cytotoxic and immunomodulatory effects, vitamin C is a major regulator of the epigenetic system of hematopoietic cells. Ascorbate is a critical cofactor for the Ten-Eleven Translocation (TET) family of DNA demethylating enzymes and for Jumonji-type histone demethylases, which help to establish regular DNA and histone methylation (Yue & Rao, 2020). In hematopoietic stem cells, sufficient levels of vitamin C allow for demethylation of tumor suppressor genes as well as facilitate proper differentiation. A TET2 loss-of-function mutation and vitamin C deficiency (a common lesion in AML) result in abnormal hypermethylation of DNA, differentiation dysfunction and, ultimately, stem/progenitor cell self-renewal (Zhang et al., 2023; Ottesen et al., 2023). In recent works, it was found that the restoration of vitamin C could enhance TET2 activity and DNA demethylation even in cells with TET2 mutations, and thus resuscitate repressed genes responsible for inhibiting leukemogenesis (Taira et al., 2023). Accordingly, ascorbate deficiency has also been found to enhance quiescence and self-renewal ability of hematopoietic stem cells in mice, thus favoring malignant transformation (Salih & Kadhim, 2024). On the other hand, the use of vitamin C reversibly reprograms these cells and restores normal differentiation pathways and genomic stability. With respect to epigenetic effects, the antioxidant/genoprotective effects of vitamin C correlate with lower frequencies of micronucleated polychromatic erythrocytes (MN-PCEs), as well as in vivo positive responses to genotoxic stress on marrow proliferative indices (e.g., PCE/NCE) to highlight its potential as a chromosomal damage reducer in erythroid precursor groups (Bo et al., 2024). Vitamin C has additionally been shown to synergistically work with epigenetic therapies.

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In an AML model, vitamin C supplementation markedly boosted the anti-leukemia activity of 3-deazaneplanocin A (DZNep), an inhibitor of epigenetic repressors and resulted in increased leukemic cell differentiation and death (Long et al., 2022). In addition, mechanistic studies on the benzene metabolite 1,4-benzoquinone have shown that ascorbate was able to ameliorate chromosome destruction and micronucleus development (Mishra et al., 2023), thereby further linking vitamin C biology to decreases of MN-PCEs. Nonetheless, a complete understanding of the action of vitamin C on reducing or preventing benzene-induced pre-leukemia remains elusive. Benzene-induced leukemogenesis is an appropriate experimental model to evaluate chemopreventive strategies, owing to benzene's strong activity to induce pre-leukemic alterations. Few studies to date have quantitatively tested whether vitamin C supplementation can help attenuate hematopoietic damage and leukemic progression resulting from benzene exposure. In this study, we sought to evaluate the protective effects of vitamin C in an in vivo rat model against benzene-mediated AML. Therefore, this study was designed to assess whether acute, graded doses of vitamin C could prevent or attenuate benzene-mediated hematotoxicity and pre-leukemic changes in rats. The main outcome is the mitigation of benzene-induced genotoxicity with secondary outcomes of hematologic recovery, restoration of bone marrow cellularity and the attenuation of systemic inflammatory skewing. We expected vitamin C to dose-dependently reduce genomic injury, enhance cellularity of the marrow, improve peripheral and marrow hematological correlates, and mitigate pro-inflammatory immune dysregulation in this benzene model of human leukemogenesis, lending credence for its promise as a chemopreventive modality.

2. Materials and Methods

2.1 Animals

Experiments were conducted on 8- to 10-week-old rats, whose weight ranged from 180 to 220 grams. They were kept under normal laboratory conditions consisting of a 12-hour illumination-dark cycle along with unlimited feeding and drinking facilities. All the methods strictly followed the widely accepted ethics in the use and treatment of animals, and the study protocol was approved by the Institutional Animal Ethics Committee of Cihan University-Erbil, Approval Number (CUE-REC/2025/ 08).

2.2 Chemicals

A benzene solution (Chem Lab, Belgium) was prepared by mixing one part benzene with five parts 2-propanol and five parts distilled water (D.W.) (v/v). Vitamin C (YourHealthStore.co.uk, UK) was weighed and the pure ascorbic acid powder dissolved in distilled water, to yield doses of 100, 200, 500, and 1000 mg/kg. The solution was vortexed until homogeneous.

2.3 Induction of Pre-leukemia with Benzene

Pre-leukemia was induced in rats with an intravenous dose of a solution of benzene. The solvent used was benzene, propanol, and D.W. with the ratio of 1:5:5. Each rat was infused with 0.2 ml through the tail vein every 48 hours for 4 weeks. This procedure was adjusted to allow for the monitoring of normal hematological variations as well as genotoxic changes that are precursors for pre-leukemic conditions. Care was taken to monitor the subject all the way through the study for any changes in physiological as well as behavioral responses (Ola et al., 2022).

2.4 Experimental Design

Rats were randomly allocated to six groups (G1-G6) to examine the dose-dependent effects of ascorbic acid (vitamin C) on benzene-induced pre-leukemic alterations. The control group (G1) was given distilled water (D.W.) orally for the entire 4-week study. The benzene group (G2) received pre-leukemia induction as detailed in Section 2.3, tail-vein infusion of 0.2 mL of a 1:5:5 benzene:2-propanol:D.W. solution every 48 hours for four weeks. Groups G3-G6 underwent the same benzene regimen as G2 and additionally received oral vitamin C once daily for four consecutive weeks at the following doses: G3, 100 mg/kg/day; G4, 200 mg/kg/day; G5, 500 mg/kg/day; and G6, 1000 mg/kg/day. All remaining housing, handling, and procedural conditions were kept identical across groups.

2.5 Bone Marrow Smear Preparation

After the 4-week treatment period, rats were euthanized by cervical dislocation. Bone marrow was flushed with phosphate-buffered saline (PBS; pH 7.2) supplemented with 50% fetal bovine serum (FBS), and the isolates were thoroughly washed. The resulting suspension was centrifuged at 3000 rpm for 10 minutes, after which the pellet was resuspended in PBS for smear preparation. A

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minimum of five slides was prepared per specimen. Smears were air-dried for 10 minutes, fixed in methanol, and stained with May-Grünwald and Giemsa (20 minutes each). Slides were then rinsed, allowed to dry completely, and examined under a light microscope (Asita & Molise, 2011).

2.6 Micronucleus Assay

For evaluating benzene-related chromosomal injury in rats, micronucleus assay was applied. Bone marrow was flushed off of femurs, washed first with 50% FBS, then PBS, and centrifuged at 3000 rpm for 10 min. The pellet was resuspended in PBS, and a small aliquot was prepared to generate smears on sterile slides of glass prewet with a spreading droplet. Smears were air-dried at room temperature, then set in methanol for 10 min, and stained using May-Grünwald and Giemsa (1:1) consecutively for 20 min. Slides were washed in phosphate buffer, air dried and subjected to light microscopy with a 100× oil immersed objective. 1000 PCEs per rat were evaluated. Assay readouts can be defined as follows:
The percentage of PCEs (PCE%) could be computed as PCE ÷ (PCE + NCE) * 100, and the percentage of NCEs as NCE ÷ (PCE + NCE) * 100. MN-PCE frequency was calculated by counting micronucleated PCEs among a pool of 1000 PCEs and represented as (MN-PCE ÷ total PCE) * 1000 (per 1000 cells). MN-NCEs were then counted among 1000 NCEs, in a similar way. The relative abundance of erythroid subpopulations was investigated through PCE fractions as PCE ÷ (PCE + NCE) × 100 (Sen et al., 2010).

2.7 Complete Blood Count (CBC)

He et al. (2017), whole-blood samples of rats were collected and processed on an automated hematology analyzer, to create a CBC profile. The panels included WBC, NEU, LYM, MON, EOS, BAS, hemoglobin (Hb), packed cell volume (PCV), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC). The platelet measures included PLT and platelet indices (mean platelet volume (MPV), platelet distribution width (PDW), plateletcrit (PCT), platelet large cell ratio (P-LCR), platelet large cell count (P-LCC)), along with derived ratios LYM/MON, NEU/LYM, NEU/MON, MPV/PLT, PLT/LYM, WBC/MPV, RDW/PLT, and SII. Differential leukocyte counts were calculated automatically to obtain the relative percentages of NEU, LYM, MON, EOS, and BAS, resulting in an integrated measure of leukocyte distribution and systemic immunity status (He et al., 2017).

2.8 Peripheral Blood Smear

Peripheral blood smears were obtained from EDTA-anticoagulated samples taken at the end of the study. In compliance with standard hematologic practice, smears were air-dried in a desiccator, fixed in absolute methanol, and stained using the May-Grünwald-Giemsa method. Blast morphology was noted as high nuclear-to-cytoplasmic ratio, large nucleoli, finely granular chromatin that had sharp margins, and intensely basophilic cytoplasm. After oil immersion at 1000×, 100 leukocytes of the smear were counted for the percentage of blasts. To improve credibility and reduce inter-observer variability, two observers independently counted leukocytes. Protocols followed standard clinical and laboratory hematology guidelines (Dai et al., 2021).

2.9 Statistical Analysis

Data were analyzed through one-way ANOVA and Tukey's post hoc test for multiple comparisons. The results are expressed as mean ± SEM. Normality and homogeneity of the data were checked before making any inferences. Graphs were generated as bar charts using GraphPad Prism (version 9.0.0). The p value for significance was considered as less than 0.05.

3. Result

3.1 Micronucleus Assay

Benzene exposure caused a pronounced suppression of %PCE and a reciprocal rise in %NCE as described in Figure 1 compared to the control group (***$p < 0.0001$), indicating strong bone-marrow cytotoxicity. Co-treatment with vitamin C (100, 200, 500, and 1000 mg/kg) significantly increased %PCE and decreased %NCE compared to the benzene group (***$p < 0.0001$). The response was dose dependent: higher doses of vitamin C produced greater correction; 1000 mg/kg restored both indices closer to the control levels. Figure 2 shows the impact on genotoxic markers. Benzene is also highly significant compared with controls, significantly reducing the PCE/NCE ratio and raising %MN-PCE and %MN-NCE (***$p < 0.0001$), suggesting

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high genotoxicity. Compared with benzene, vitamin C at all doses significantly improved the PCE/NCE ratio and minimized micronuclei formation ($p < 0.0001$). MN-PCE decreased in a dose-dependent manner, and 1000 mg/kg was close to baseline, while all doses of vitamin C normalized MN-NCE. These data all suggest that vitamin C attenuates benzene-induced cytotoxicity and genotoxicity in a graded, dose-dependent manner.

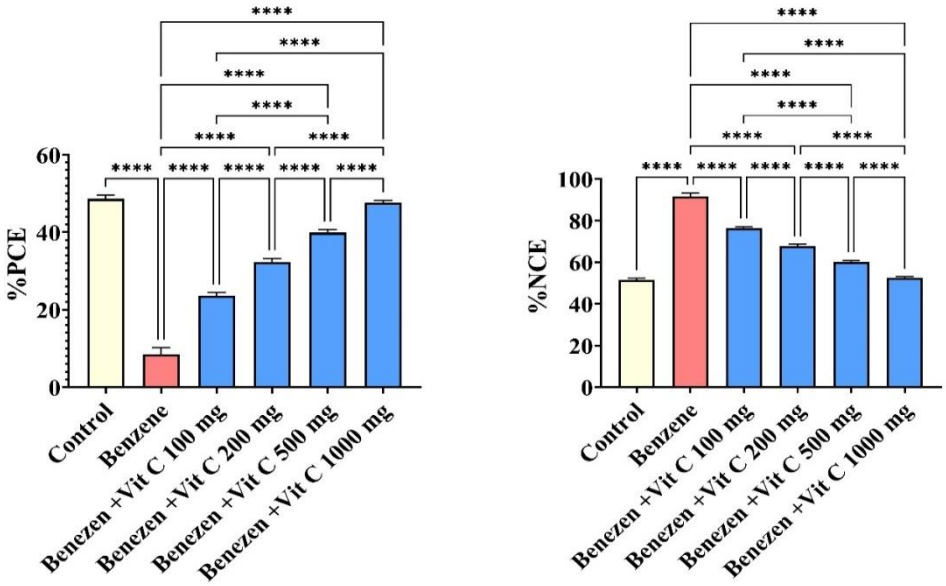

Figure 1: Effects of different doses of Vitamin C on %PCE and %NCE in Benzene-Exposed Rat.

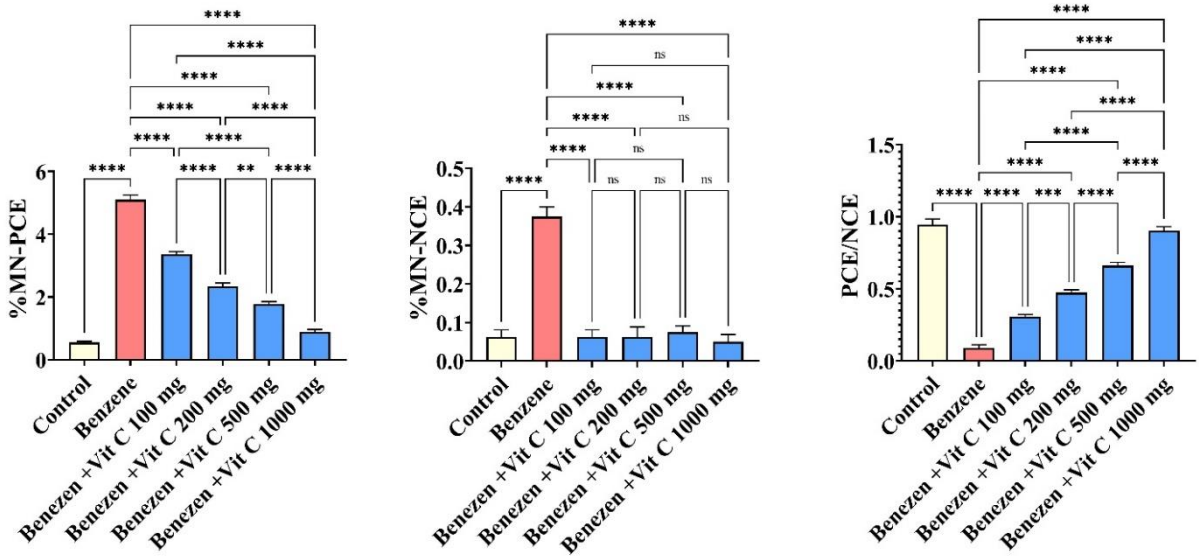

Figure 2: Effects of different doses of Vitamin C on %MN-PCE, %MN-NCE, and PCE/NCE ratio in Benzene-Exposed Rat.

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3.2 Alterations in Differential Leukocyte Count

As shown in Table 1, benzene exposure markedly disrupted the leukogram relative to control, with a significant fall in total WBCs (11.93 → 7.54 ×10^3/µL) and reductions in LYM and MON, accompanied by pronounced neutrophilia and basophilia; EOSs were also elevated (significance as indicated by * vs control). Administration of vitamin C to benzene-treated rats improved these abnormalities in a dose-responsive manner. At 100-500 mg/kg, WBCs recovered toward control (10.92, 8.97, 10.07 ×10^3/µL), LYM and MON counts increased, and the benzene-induced rises in NEU and BAS were significantly attenuated. EOS was markedly suppressed to below control across these doses. The 1000 mg/kg dose produced the strongest reduction of NEU and BAS (1.52 and 0.026 ×10^3/µL, respectively) but was associated with lower total WBCs and LYM than the 100-500 mg/kg groups. Collectively, these findings indicate that vitamin C mitigates benzene-induced leukocyte disturbances, with low to moderate doses (100-500 mg/kg) providing the most balanced restoration toward control levels.

Table 1: Effects of different doses of Vitamin C on differential leukocyte count and blast cells in Benzene-Exposed Rat.

Parameters	Control	Benzene	Benezen +Vit C 100 mg	Benezen +Vit C 200 mg	Benezen +Vit C 500 mg	Benezen +Vit C 1000 mg
WBCs count (Cells x 10^3 /µL)	11.93 ± 0.9967	7.535 ± 0.7221*	10.92 ± 1.024##	8.973± 0.5533	10.07± 0.8976#	6.111± 1.000
NEU count (Cells x 10^3 /µL)	3.074 ± 0.3156	4.042 ± 0.7045	4.000± 0.4259##	3.485 ± 0.4516#	3.129± 0.4325	1.516± 0.3313**
LYM count (Cells x 10^3 /µL)	7.936 ±0.8087	5.535 ± 0.5483	6.576± 0.8696	6.206 ± 0.4687	6.366± 0.6273	5.283± 0.4033
MON count (Cells x 10^3 /µL)	0.8045 ± 0.1967	0.4165 ± 0.0532***	0.4250± 0.1093***	0.6975 ± 0.1608***	0.5025± 0.05769***	0.5400± 0.1036***
EOS count (Cells x 10^3 /µL)	0.1065 ± 0.0334	0.1354 ± 0.0489***	0.03000± 0.005669***	0.0100± 0.0037***	0.008750 ±0.006105***	0.01125± 0.004407***
BAS count (Cells x 10^3 /µL)	0.0154 ± 0.0083	0.1265 ± 0.0478**	0.06375± 0.03438	0.05750 ± 0.01578*	0.04250± 0.007734	0.02625± 0.003239**
%Blast Cells	0.000 ± 0.000	35.00 ± 1.402***	26.50±0.8660***###	12.25 ± 0.5901***###	8.125± 0.7181***##	3.625± 0.5957***

Star sign (*) denotes that Benzene is compared with the control and treatment groups, while # denotes that Vitamin C low doses are compared with High dose treatment.

WBCs count: White Blood Cell count; NEU: Neutrophils; LYM: Lymphocytes; MON: Monocytes; EOS: Eosinophils; BAS: Basophils

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3.3 Differential Leukocyte Ratios

Figure 3 illustrates the effects of graded vitamin C 100-1000 mg/kg on leukocyte-derived ratios LYM/MON, NEU/LYM, and NEU/MON in the benzene model. Benzene exposure significantly reduced LYM/MON (***p < 0.001) and NEU/MON (****p < 0.0001) compared with the control group, while NEU/LYM remained largely unchanged, non-significant (ns). Vitamin C treatment improved these disturbances: LYM/MON was significantly restored versus benzene at 100 and 200 mg/kg (*p < 0.05 for each), with higher doses showing comparable levels and no meaningful inter-dose differences ns. NEU/MON was likewise elevated by vitamin C 100 mg/kg and 200 mg/kg produced significant improvements over benzene (*p < 0.05 and **p < 0.01, respectively), whereas 500-1000 mg/kg brought the ratio toward control with mostly non-significant differences among treated groups ns. NEU/LYM showed minimal modulation overall, differing significantly only at 1000 mg/kg (**p < 0.01 vs selected groups), while other comparisons were ns. These findings indicate that vitamin C mitigates benzene-induced alterations in leukocyte balance, with LYM/MON and NEU/MON emerging as the most responsive indicators of treatment efficacy.

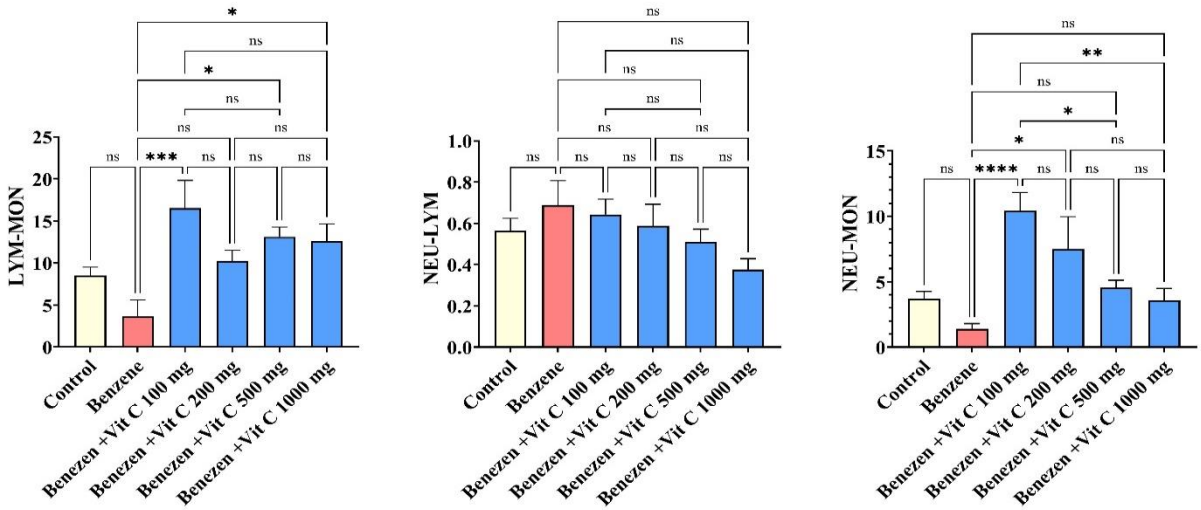

Figure 3: Effects of different doses of Vitamin C on Leukocyte Ratios (LYM/MON, NEU/LYM, and NEU/MON) in Benzene-Exposed Rats.

3.4 Platelet Count and Indices

As shown in Table 2, benzene exposure significantly reduced PLT count, MPV, PDW, and %PCT relative to the control group, while elevating PLT/LYM, RDW/PLT, and SII, indicating PLT dysfunction with inflammatory skewing. Oral vitamin C (100-1000 mg/kg) improved these abnormalities: PLT count and %PCT were restored toward or above control values, MPV and PDW increased compared with benzene, MPV/PLT and RDW/PLT declined, and WBC/MPV improved at lower doses, although PLT/LYM remained relatively high, particularly at the highest dose. Results from Figure 4 further support these observations: benzene markedly decreased PLCC compared with control (*p < 0.05), while %PLCR was not different from control ns. Vitamin C significantly raised %PLCR at 200 mg (p < 0.01) and 500 mg (*p < 0.001), with a modest increase at 1000 mg (p < 0.05), and robustly restored PLCC across treatment groups versus benzene (significant increases, p < 0.05 or p < 0.01, as annotated), with no meaningful differences among vitamin C doses ns. Collectively, these findings indicate that vitamin C effectively mitigates benzene-induced PLT abnormalities and improves PLT morphology-related indices, with intermediate high doses showing the most consistent recovery.

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Table 2: Effects of different doses of Vitamin C on PLT count and its indices In Benzene-Exposed Rat

Parameters	Control	Benzene	Benezen +Vit C 100 mg	Benezen +Vit C 200 mg	Benezen +Vit C 500 mg	Benezen +Vit C 1000 mg
PLT count (Cells x 10^3 /µL)	435.6 ± 41.51	424.6 ± 61.74	435.6 ± 33.48	445.1 ± 60.80	524.6 ± 42.22	564.9 ± 89.90
MPV count (fL)	9.384 ± 0.2665	6.47 ± 0.0843	7.050 ± 0.2353	7.650 ± 0.3202	7.550 ± 0.2299	6.850 ± 0.2390
PDW count (fL)	12.53 ± 0.6065***	9.055 ± 0.4154	9.813 ± 0.4977	7.475 ± 0.4576	9.988 ± 1.480	7.475 ± 0.4578
%PCT	0.4056 ± 0.0454	0.2776 ± 0.0343	0.3064 ± 0.02712	0.3763 ± 0.0619	0.3939 ± 0.03058	0.4378 ± 0.03361*
MPV/PLT	0.0225 ± 0.0035	0.0188 ± 0.0034	0.01690 ± 0.001434	0.0325± 0.0173	0.015 ± 0.0018	0.02040 ±0.008611
PLT/LYM	59.24 ± 7.686	76.47 ± 8.275	75.34± 11.57	71.87±10.02	99.71± 9.419	110.4± 22.29
WBC/MPV	1.285 ± 0.1154*	1.184 ± 0.1243	1.495±0.1359 #	1.144 ± 0.0963	1.123±0.1486	0.8688±0.1784
RDW/PLT	0.0913 ± 0.0083	0.1134 ± 0.01563	0.04000 ± 0.003300	0.0700 ± 0.0414	0.03650 ±0.004687	0.07600 ± 0.04100
SII	179.3 ± 25.07	290 ± 219.1	288.7±20.70	322.8 ± 51.35	307.1±15.89	314.7± 16.13

Star sign (*) denotes that Benzene is compared with the control and treatment groups, while # denotes that Vitamin C low doses are compared with High dose treatment.

PLT: Platelet count; MPV: Mean Platelet Volume; PDW: Platelet Distribution Width; PCT: Plateletcrit; MPV/PLT: Mean Platelet Volume to Platelet Ratio; PLT/LYM: Platelet to Lymphocyte Ratio; WBC/MPV: White Blood Cell to Mean Platelet Volume Ratio; RDW/PLT: Red Cell Distribution Width to Platelet Ratio; SII: Systemic Immune-Inflammation Index.

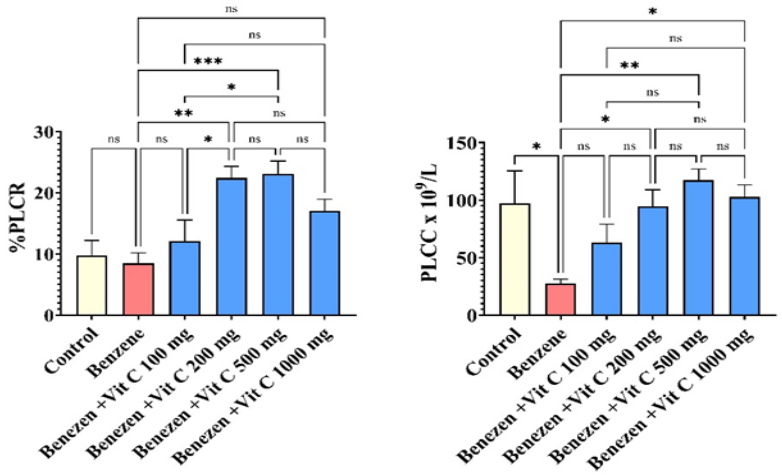

Figure 4: Effects of different doses of Vitamin C on Platelet Indices (%PLCR and PLCC) in Benzene-Exposed Rats.

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3.5 Microscopic Examination of Blood Cells

The proportion of blast cells in the peripheral blood smear of the group exposed to benzene was markedly increased to 35-37% in Figure 5, suggesting significant pre-leukemic transformation. In comparison, the control group had no detectable blasts, as characteristic of a normal hematopoietic profile. Vitamin C administration confirmed a dose-dependent correction: blast was reduced to 26–28% by 100 mg/kg, to 15–17% by 200 mg/kg, to 7-9% by 500 mg/kg, and to 3-4% by 1000 mg/kg, each having a dramatically greater level of reduction compared with the benzene group (*p < 0.0001). Pairwise comparisons for each vitamin C dose revealed other major differences (-***p, as noted), with the highest dose approaching a near basal level. These findings reveal that elevated doses of vitamin C efficiently inhibit benzene-induced pre-leukemic blasts in peripheral blood, with the highest normalization at 1000 mg/kg. The microscopic morphology of the blast cells (as shown on the peripheral blood smears) is depicted in Figure 6.

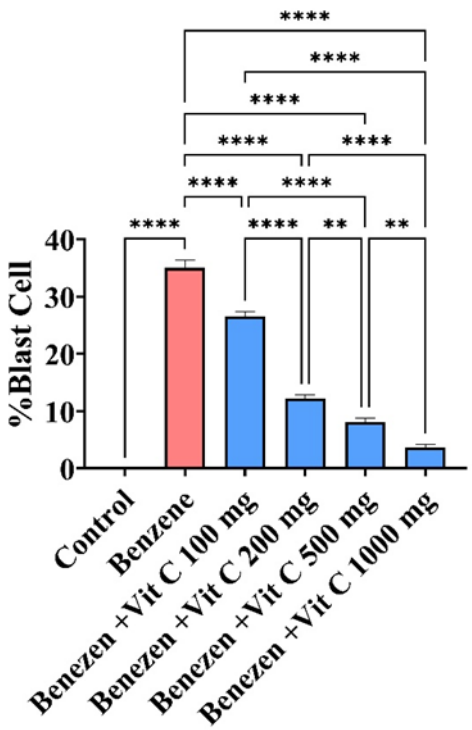

Figure 5: Effects of different doses of Vitamin C on the Percentage of Blast Cells in Peripheral Blood smear of Benzene-Induced pre-Leukemia Rat.

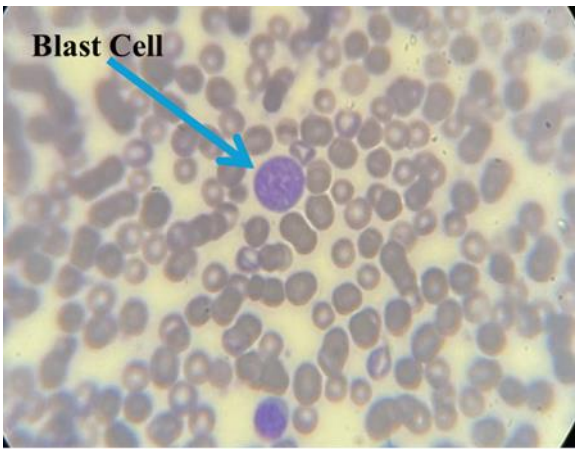

Figure 6: Morphological Appearance of Blast Cells in Peripheral Blood Smear Under Light Microscopy 100x using May Grunwald and Giemsa Stain.

4. Discussion

In this study, benzene exposure generated significant genotoxic pressure in the marrow, reflected by increased numbers of micronucleated erythrocytes and decreased erythropoiesis. Benzene markedly decreased PCE percentage and PCE/NCE ratio, which is a measure of marrow proliferative efficiency, implying a decreased generation of new erythroid cells. This is consistent with the confirmed myelotoxic profile of benzene and its reactive quinone metabolites, which cause DNA double-strand breaks and chromosomal damage in hematopoietic stem cells resulting in formation of micronucleus on an appropriate (erythroid precursors in this study) basis (Spatari et al., 2021). Indeed, elevated micronucleus frequencies in bone marrow or blood are a typical feature of benzene's genotoxicity and eventual evolution into leukemic transformation (Golabi-Habashi et al., 2021). In addition, vitamin C co-administration dose-dependently attenuated these genotoxic effects. Among all the ascorbic acid doses, the frequency of micronucleated PCEs and NCEs was significantly reduced by vitamin C addition. Mechanistically, vitamin C's strong antioxidant activity is likely to mediate scavenging of ROS and free radicals released by the metabolism of benzene, thus, reducing the likelihood of DNA lesions and chromosomal disruption (Mishra et al., 2023). Several studies indicated antioxidant compounds to moderate clastogenicity caused by benzene, such as

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vitamin C–vitamin E supplementation that reduced micronucleus formation and chromosome aberrations by the metabolite 1,4-benzoquinone of benzene in vivo (Mishra et al., 2023). In this regard, one plant flavonoid quercetin (a polyphenolic antioxidant) showed significant reduction of benzene-enriched micronuclei in mouse bone marrow, as observed in the protective effect with vitamin C (Golabi-Habashi et al., 2021). Vitamin C not only reduces oxidative DNA damage but may even promote DNA repair in order to preserve genomic stability through a cofactorial role to epigenetic enzymes (Zhang et al., 2023). Importantly, ascorbate is necessary for ten-eleven translocation (TET) dioxygenase activity, inducing demethylation and genomic integrity of DNA in hematopoietic cells that promotes regenerating DNA (Zhang et al., 2023). Vitamin C restored %PCE and PCE/NCE toward normal cellularity and proliferative capacity in the bone marrow, previously severely suppressed by benzene. This maintenance of erythropoiesis supports findings that ascorbate-adequate conditions promote healthy hematopoietic stem cell differentiation and prevent defective differentiation and genomic instability in bone marrow (Guan et al., 2020). In this manner, the vitamin C seemed to shield the erythroid lineage adequately from the cytotoxic and genotoxic onslaught of benzene, preventing the genotoxic damage (micronuclei) and marrow aplasia that results in leukemic progression. Benzene induced a marked state of leukopenia in our rat model wherein total white blood cell count was reduced by roughly a third and leukocyte differential was distorted. This pattern is consistent with benzene's known immunosuppressive effects in exposed humans and animals: the toxin preferentially attacks bone marrow progenitors, often resulting in pancytopenia with a significant decline in LYM. The lineage with the strongest suppression in our benzene group, LYM demonstrated clear lymphopenia and MON was also significantly decreased (Guo et al., 2021). The susceptibility of lymphoid cells to benzene has been well established thus far: previous studies have demonstrated preferential inhibition of LYM (including T, B, and NK cells) due to benzene toxicity against adaptive immune cells. In contrast, exposed animals to benzene presented NEU in greater proportion (and probably absolute counts), as well as relative eosinophilia and basophilia. One explanation might be that benzene caused a pro-inflammatory or stress response that stimulated granulocyte mobilisation. Marrow injury may have led to cytokine-directed release of NEU and other granulocytes into the circulation (e.g., interleukin-1, interleukin-8, and G-CSF), despite diminished lymphoid production (Giardini et al., 2023). Similar dual effects have been described in exposed cohorts, where high doses of benzene generally induce neutropenia, with lower doses or subacute exposures likely leading to neutrophilia and increased basophils with LYM depletion. And benzene induced tissue damage with oxidative stress probably induced mild inflammatory state, which accounts for the rise in NEUs. In particular, co-administration of vitamin C largely normalized leukocyte profile. Total WBC numbers recovered in vitamin C classes suggesting that ascorbate prevented the benzene-induced leukopoiesis bone marrow failure (Mishra et al., 2022). In particular D4-based vitamin C helped in preserving LYM and MON and therefore mediated against the immunosuppressive effects of benzene. LYM may be preserved as vitamin C has shown to be an immune cell survivor and enhancer; ascorbate can favour LYM proliferation and function mainly by inhibiting oxidative apoptosis and also by producing other antioxidants, such as glutathione from leukocytes (Méndez López et al., 2024). Vitamin C also acts to moderate the chronic inflammatory signaling mechanism caused by benzene. Vitamin C reduces pro-inflammatory cytokines through its scavenging of ROS and modulation of pathways through which Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is promoted, thereby preventing abnormal granulocyte expansion and general immune injury. The elevated NEU, EOS, and BAS in our study that were the product of benzene exposure were significantly attenuated by vitamin C suggesting a re-balancing of myeloid output. The net result was a return of immune homeostasis: vitamin C-treated rats exhibited a more physiologic distribution of NEU, LYM, and other leukocytes, contrasted with a distorted differential observed with untreated benzene toxicity. Such results emphasize vitamin C as a source of immunosafety, maintaining white cell proliferation and diversity in the context of benzene-induced myelotoxic and immunotoxic effects (Sasidharan Nair & Huehn, 2024; Méndez López et al., 2024). In line with the above trends, exposure to benzene significantly tilted critical leukocyte count ratios, and vitamin C corrected for these

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fluctuations. In benzene-only rats, NEU/LYM ratio was possibly elevated, and LYM/MON ratio was abnormally low pattern showing systemic inflammation and immunological dysregulation. The NEU/LYM ratio is a sensitive index of stress and inflammation; increasing the NEU/LYM ratio demonstrates neutrophilia with lymphopenia and has been linked with chronic inflammation and worse prognoses in hematologic disorders (Golonko et al., 2024). A high NEU/LYM ratio in our benzene-treated animals suggested an inflammatory predominance and severe LYM loss. Another immunologic marker commonly used in infection and cancer is the LYM/MON ratio; the LYM/MON ratio can be depressed (due to lymphopenia and/or relative monocytosis), associated with impaired adaptive immunity and observed in conditions of bone marrow stress. The simultaneous depletion of LYM and (to a lesser extent) MON with benzene as the dual effect likely decreased LYM/MON in this study, supporting previous work indicating that LYM decreased for benzene-exposed individuals in association with abnormal MON counts (Cordiano et al., 2022). Similarly, NEU/MON ratio was disturbed by benzene, which was associated with disproportionate NEU decrease over MON count. These are abnormal ratios which indicate that the drug benzene impels the immune system to become pro-inflammatory, myeloid-skewed state, yet at the same time suppresses lymphoid elements. Vitamin C treatment could correct these imbalances. Ascorbic acid treatment shifted the NEU/LYM ratio, LYM/MON, and NEU/MON ratios back to the normal range, indicating a return to a balanced balance of innate and adaptive immune cells. Vitamin resulted in increased LYM counts (raise the LYM/MON ratio), and tempered NEU excess (reduce NEU/LYM), showing its ability to restore immune homeostasis. Not only is this normalization of leukocyte ratios statistically significant but it is also biologically significant: balanced ratios also correspond to resolved inflammatory state and improved immunocompetence (Méndez López et al., 2024). This immune rebalancing consistent with its known anti-inflammatory and immunomodulatory properties can also be derived from vitamin C. It has a role in limiting the oxidative stress response and in limiting inflammatory pathways, which can prevent NEU overactivation but also promote the maintenance of lymphocyte stability (Morante-Palacios et al., 2022). Vitamin C also facilitates phagocyte function, and may promote monocyte/macrophage resistance to toxic stress and thus maintain optimal monocyte numbers. Between them, the normalization of NEU/LYM, LYM/MON, and other indices with vitamin C showed that this antioxidant is not only protective against quantitative cell losses but also a source of qualitative immune disturbances in the vitamin C treated rats. The immune-cell ratios of vitamin co-treated rats were in close vicinity to the healthy controls, suggesting that ascorbate successfully attenuated benzene-dependent chronic inflammation and immune disequilibrium (Nathan et al., 2024). These data solidify that vitamin C maintains immunologic equilibrium when introduced toxins might threaten to upset it. Benzene exposure additionally resulted in marked thrombocytopenia and altered PLT indices, suggesting injury to the megakaryocytic lineage in a pro-inflammatory environment. PLT counts (PLT) and plateletcrit (PCT) - the blood volume fraction occupied by PLT - were considerably reduced in benzene-only rats, which coincides with the myelotoxic inhibition of PLT production by benzene. It is well-established that myelosuppression can reduce circulating PLT in tested animal studies as well as in exposed worker populations (Huang et al., 2024). We further observed changes in PLT morphology indices: benzene-treated animals had lower mean platelet volume (MPV) and higher platelet distribution width (PDW), consistent with a greater tendency toward smaller, perhaps older, PLT and a diverse size profile. In thrombocytopenic scenarios, decreased MPV could signify impaired megakaryocyte function whereby the marrow doesn't release the normally large, younger PLT owing to either direct megakaryocyte toxicity or attenuated thrombopoietin signaling. Benzene-mediated oxidative injury of the marrow, which apparently leads to impaired production of megakaryocytes, is another factor leading to decreased and morphologically abnormal PLT (Phadke et al., 2022). The benzene group also showed elevated pro-inflammatory hematologic ratios involving PLT; for example, PLT/LYM increased because LYM decreased more than PLT. An increased PLT/LYM ratio is generally considered to be a marker of systemic inflammation or stress and is associated as well with disease severity of inflammatory and malignant states (Binsaleh et al., 2024).

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Likewise, the SII across NEU, LYM, and PLT (NEU × PLT / LYM) had benzene considerably increased. Higher SII was caused by both the combination of thrombocytopenia (lower PLT) with profound lymphopenia and neutrophilia in our benzene group. While PLT were decreased, the critically low lymphocyte counts and relative neutrophilia increased the SII. This profile is consistent with bone marrow stress and associated inflammation in which benzene promotes significant NEU and PLT turnover and scarce LYM. The observed improvement in PLT dysregulation along with inflammatory parameters was attributed to vitamin C concurrent treatment. Ascorbic acid sustains PLT production: PLT in vitamin C-treated groups recovered to near-control or even beyond baseline following some concentrations reflecting resolved thrombopoiesis (He et al., 2022). The PLT improvement was dose dependent with higher vitamin C doses providing better protection against benzene-induced thrombocytopenia. This restoration may be attributed to vitamin C protecting megakaryocytes against oxidative apoptosis and supporting their maturation. Vitamin C also participates in collagen synthesis and bone marrow stromal architecture, which can assist in maintaining the niche needed for megakaryopoiesis. Moreover, its antioxidant power could serve to prevent the decline of the thrombopoietic milieu, and prevent the autoimmunity-driven PLT attacks that result from oxidative stress (Aliyu et al., 2017). In line with increased PLT output, plateletcrit was normalized by vitamin C, as evidenced by the normalization of MPV and PDW in vitamin C co-treated rats, indicating more normal PLT morphology. Increased MPV (from its abnormally low values on benzene by itself) suggest new, larger PLT were formed, signalling a bone marrow recovery. Moreover, PDW decreasing toward control suggests an even distribution of PLT-size to suggest stable PLT production and not anisocytosis of extreme size. These findings corroborate other protective measures; for instance, natural antioxidants have been proven to reinstate PLT counts and reduce morphological changes in toxin-exposed animals (Ola et al., 2022). Vitamin C also significantly decreased pathological elevation of PLT-related inflammatory ratios. In vitamin C treated animals, PLT/LYM ratio decreased toward a level comparable to control values owing to elevated lymphocyte count and platelet count stabilization. This appears to be indicative of an overall suppression of inflammation in the systemic system. Additionally, SII was significantly lower in vitamin C groups relative to benzene-only rats, showing a concerted reduction of NEU-driven inflammation and an improvement in LYM survival. Decreased SII and PLT/LYM in treated rats signal a less inflammatory, more immunologically stable state, which vitamin C prevented by inhibiting the pro-inflammatory signals of benzene. Combined and a new benefit of note, vitamin C maintained the thrombocytic lineage for the first time and prevented the secondary effects of thrombocytopenia that present as increased inflammatory indices. The impact of ascorbate is not only to mitigate a bleeding hazard caused by decreased platelet count because it maintains a homeostatic balance within the platelets but also to disrupt the vicious cycle of inflammation and organ dysfunction. Such results corroborate earlier research in which vitamin C or similar antioxidants protected hematologic parameters in toxic injury models for example vitamin C has been shown to ameliorate redox and reduce markers of inflammatory response in metabolic stress states (Aghaei et al., 2023), and we have found that a similar benefit between the positive treatment and hematologic inflammatory markers of benzene stress has occurred here too. Vitamin C restores PLT count and normalizes indices, such as SII, which emphasize its role in ameliorating benzene-induced systemic inflammation and coagulopathies. Arguably, one of the most salient features observed in benzene-exposed rats was the remarkable high percentage of circulating blast cells observed (35% blasts vs 0% circulating blast cells in controls). Clearly, such a surge indicates a leukemogenic effect of benzene, as immature myeloblasts are typically confined to the marrow and the spilling into blood indicates high levels of acute pre-leukemia or blast-phase. Benzene is an established leukemogenic agent able to induce clonal proliferation of cancerous hematopoietic cells; based on our data, benzene continued to drive a pre-leukemic disease to overt leukemic blastemia even after a 4-week exposure. Mechanistically, a reaction of reactive metabolites from benzene probably resulted in mutations of importance or chromosomal lesions in hematopoietic stem/progenitor cells (Zhao et al., 2021). In addition to benzene-induced marrow dysregulation, these genotoxic insults facilitate aberrant clones in expansion and spillover into the circulation through blasts in a similar way to pathway of

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treatment-based or toxin-based AML development, where primary DNA damage/genomic instability promotes clonal evolution of blast populations (Zhao et al., 2025). In our model, the high blast fraction in addition to cytopenias resembles an AML image, a lethal condition that, if untreated, would develop into fatal pre-leukemia. Interestingly, vitamin C co-treatment virtually arrested this leukemic cascade (Lu et al., 2023). Across the doses, peripheral blood blasts were dramatically decreased; at 1000 mg/kg, they fell to 3-4%. However, this powerful protection suggests that vitamin C interferes with one or more key steps within benzene-induced leukemogenesis, likely with the protection from the initial DNA damage and stem-cell injury that leads to malignant clones as observed in vitamin C-treated marrow with reduced micronuclei and chromosomal breaks (Liu et al., 2020). Vitamin C could lower the likelihood of the leukemic transformation when taking root by protecting the hematopoietic stem cell genome. Vitamin C may also have facilitated apoptosis or differentiation of any abnormal cells that did occur. Emerging evidence suggests that high-dose ascorbate is a pro-oxidant in cancer cells and only selectively induces cytotoxicity in malignant blasts, sparing otherwise healthy cells (Testa et al., 2021). Vitamin C at pharmacologic concentrations can yield hydrogen peroxide near cancer cells and induce pro-apoptotic pathways mediated by oxidative stress in leukemic cell lines (Tronci et al., 2021). Further, vitamin C can also reactivate silenced tumor-suppressor programs via epigenetic mechanisms; ascorbate has been shown to reactivate TET2 and other 5-methylcytosine hydroxylases, driving differentiation of myeloid blasts into mature cells (Yue & Rao, 2020; López-Moyado et al., 2024). Vitamin C is demonstrated to be synergistically involved in action by epigenetic therapies in AML models, inducing leukemic cell differentiation and death, and can induce endoplasmic reticulum stress and apoptosis directly in acute lymphoblastic leukemia cells (Das et al., 2020; Smith-Díaz et al., 2021). Thus, among our benzene-exposed rats in the model of leukemia, vitamin C likely had a dual protective effect against the development of leukemia, protecting normal hematopoietic cells from the development of malignant mutations and repressing the neoplastic clones arising. The combined result was much decreased blast burden, which effectively stopped the progression of the disease at a pre-leukemic or early stages of leukemia. This result is of great importance as circulating blast count is an important prognostic factor in pre-leukemia, therefore low blasts could improve the clinical picture (Youssef et al., 2022). Vitamin C here reduced the blast percentage almost to normal, reflecting almost complete inhibition of benzene-induced pre-leukemia. Our results are in accordance with other preventive guidelines that have been found in the literature. In a benzene-induced leukemogenesis model, one such effect is achieved with a natural antioxidant compound, kolaviron, which decreased peripheral blasts and restored bone marrow morphology (Ola et al., 2022). Vitamin C's effectiveness in our work supports the rationale that antioxidant and anti-mutagenic therapies can prevent the progression of benzene-induced bone marrow injury into fully developed pre-leukemia. The combination of antioxidant DNA protection, anti-inflammatory properties, and active anti-leukemic functions attributed in vitamin C forms a mechanistic foundation for its remarkable contribution to suppressing blasts. In addition, the involvement of vitamin C in maintenance of hematopoietic stem cell (HSC) function is probably crucial: recent investigations showed that mice lacking ascorbate exhibit enhanced self-renewal of HSCs and are biased towards undifferentiated expansion (Guan et al., 2020). That could lead to clonal evolution of pre-leukemic cells. Through providing sufficient availability of ascorbate, our treated rats may have exhibited more regulated HSC growth and differentiation, possibly limiting uncontrolled expansion of blasts. These results illustrate that vitamin C acted effectively as a chemopreventing agent in the benzene pre-leukemia model. It preserved the normal bone marrow architecture and function so that the underlying cascade of processes causing malignant blast proliferation was severed. The implications of these new findings are substantial: an easily accessible, low-toxicity agent such as vitamin C may dramatically decrease the likelihood of environmental leukemogens appearing as pre-leukemia. The dramatic reduction of blast cells that was achieved in conjunction with the normalized blood count and cytogenetic stability suggested a potent safeguard of vitamin C in the hematopoietic system against benzene-induced pre-leukemic transformation. This protective effect is remarkable in light of a rising number of studies investigating high-dose vitamin C as adjunctive cancer

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therapy, and our study findings carry our findings to an extended view of cancer prevention in a risky exposure paradigm (Luchtel et al., 2020; Zhao et al., 2025). In summary, the review of our results indicates that vitamin C's protective mechanism exists on multiple fronts, biological, cellular, and systemic to reduce path of the chronic response to the various detrimental effects induced by benzene toxicity and prevent the path toward pre-leukemia.

5. Conclusion

For the first time, this study showed that Vitamin C, at 100-1000 mg/kg doses could counteract the benzene-induced bone marrow toxicity and genotoxicity, along with pre-leukemic changes in rats. The finding indicated that Vitamin C increases the rate of erythropoiesis by increasing PCE counts, decreasing the percentage of NCE, and restoring the PCE/NCE ratio, while significantly reducing micronuclei formation (MN-PCE, MN-NCE) with 1000 mg/kg, approaching control-level genotoxicity values. Vitamin C notably reinstated immune homeostasis by rebalancing leukocyte defects: white blood cell and LYM counts significantly increased, neutrophils and basophils were modified, and immunological ratios (e.g., LYM/MON, NEU/MON) reached normal physiological balance. Furthermore, platelet indices (PLT, PCT, MPV, PDW) showed increased megakaryopoiesis and decreased inflammatory activity, while intermediate doses (100–500 mg/kg) resulted in maximal balanced hematologic recovery.

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