

Hematoprotective and Genoprotective Effects of Graded Vitamin C Supplementation in Benzene-Induced Pre-Leukemic Rats

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Research Article

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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 ($\downarrow$ %PCE and PCE/NCE; $\uparrow$ %NCE, MN-PCE, MN-NCE; $p < 0.0001$), leukopenia (WBC $\downarrow$ 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. 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 [1, 2]. 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 [3, 4]. 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 leukaemia [5–7]. 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-PCEs (as well as the PCE/NCE ratio) is widely used to detect such DNA damage in vivo and to evaluate bone marrow proliferation. It has been reported that exposure to benzene caused the measurement of micronucleus frequencies to increase a good deal in rodent bone marrow and peripheral blood due to genomic instability due to leukemic transformation [8, 9]. 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 leukemic-associated risk associated with exposure. So far, no single chemopreventive agent is sufficiently effective to protect against benzene hematotoxicity [10, 11]. 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 [12, 13]. 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 [14, 15]. 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 [16, 17]. 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 [18, 19]. 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 [20, 21]. Vitamin C, for example, induced apoptosis in acute lymphoblastic leukemia cells by accentuating endoplasmic reticulum stress following in vivo administration [22, 23]. 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 [24, 25]. 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 [26, 27]. 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 [28]. 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 [26, 29]. Accordingly, ascorbate deficiency has also been found to enhance quiescence and self-renewal ability of hematopoietic stem cells in mice, thus favoring malignant transformation [28, 30]. On the other hand, the use of vitamin C reversibly reprograms these cells, restoring 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 [31, 32]. Vitamin C has additionally been shown to synergistically work with epigenetic therapies. 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 [33]. In addition, mechanistic studies on the benzene metabolite 1,4-benzoquinone have shown that ascorbate was able to ameliorate chromosome destruction and micronucleus development [34, 35], 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.

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 was 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 [36].

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 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 [37].

2.6 Micronucleus Assay

For evaluating benzene-related chromosomal injury in rats, the 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 glass slides prewetted 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 $PCE \div (PCE + NCE) * 100$, and the percentage of NCEs as $NCE \div (PCE + NCE) * 100$. MN-PCE frequency was calculated by counting micronucleated PCEs among a pool of 1000 PCEs and represented as $(MN-PCE \div total\ PCE) * 1000$ (per 1000 cells). MN-NCEs were then counted among 1000 NCEs, similarly. The relative abundance of erythroid subpopulations was investigated through PCE fractions as $PCE \div (PCE + NCE) \times 100$ [38].

2.7 Complete Blood Count (CBC)

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 [39].

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 [40].

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 $\pm$ 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 Fig. 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 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.

Rat.

ratio in Benzene-Exposed Rat.

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 ($11.93 \rightarrow 7.54 \times 10^3/\mu\text{L}$) 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 ($10.92, 8.97, 10.07 \times 10^3/\mu\text{L}$), 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 $0.026 \times 10^3/\mu\text{L}$, 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 / μ L)	11.93 $\pm$ 0.9967	7.535 $\pm$ 0.7221*	10.92 $\pm$ 1.024##	8.973 $\pm$ 0.5533	10.07 $\pm$ 0.8976#	6.111 $\pm$ 1.000
NEU count (Cells x 10 ³ / μ L)	3.074 $\pm$ 0.3156	4.042 $\pm$ 0.7045	4.000 $\pm$ 0.4259##	3.485 $\pm$ 0.4516#	3.129 $\pm$ 0.4325	1.516 $\pm$ 0.3313**
LYM count (Cells x 10 ³ / μ L)	7.936 $\pm$ 0.8087	5.535 $\pm$ 0.5483	6.576 $\pm$ 0.8696	6.206 $\pm$ 0.4687	6.366 $\pm$ 0.6273	5.283 $\pm$ 0.4033
MON count (Cells x 10 ³ / μ L)	0.8045 $\pm$ 0.1967	0.4165 $\pm$ 0.0532***	0.4250 $\pm$ 0.1093***	0.6975 $\pm$ 0.1608***	0.5025 $\pm$ 0.05769***	0.5400 $\pm$ 0.1036***
EOS count (Cells x 10 ³ / μ L)	0.1065 $\pm$ 0.0334	0.1354 $\pm$ 0.0489***	0.03000 $\pm$ 0.005669***	0.0100 $\pm$ 0.0037***	0.008750 $\pm$ 0.006105***	0.01125 $\pm$ 0.004407***
BAS count (Cells x 10 ³ / μ L)	0.0154 $\pm$ 0.0083	0.1265 $\pm$ 0.0478**	0.06375 $\pm$ 0.03438	0.05750 $\pm$ 0.01578*	0.04250 $\pm$ 0.007734	0.02625 $\pm$ 0.003239**
%Blast Cells	0.000 $\pm$ 0.000	35.00 $\pm$ 1.402***	26.50 $\pm$ 0.8660***###	12.25 $\pm$ 0.5901***###	8.125 $\pm$ 0.7181***##	3.625 $\pm$ 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

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.

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 Fig. 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.

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.

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 Fig. 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 Fig. 6.

Peripheral Blood smear of Benzene-Induced pre-Leukemia Rat.

Light Microscopy 100x using May Grunwald and Giemsa Stain.

4. Discussion

Benzene exposure induced substantial genotoxic stress in the bone marrow, as shown by increased micronucleated erythrocytes and impaired erythropoiesis. The marked reduction in PCE percentage and PCE/NCE ratio indicates suppressed marrow proliferative efficiency and reduced production of new erythroid cells. This finding is consistent with the known myelotoxicity of benzene and its reactive quinone metabolites, which induce DNA double-strand breaks and chromosomal damage in hematopoietic stem cells, leading to micronucleus formation in erythroid precursors [41, 42]. Elevated micronucleus frequency in bone marrow or blood is a recognized feature of benzene genotoxicity and progression toward leukemic transformation [43, 44]. Vitamin C co-administration dose-dependently attenuated these effects, significantly reducing micronucleated PCEs and NCEs. This protective action is likely related to the strong antioxidant activity of vitamin C, which scavenges ROS and free radicals generated during benzene metabolism, thereby limiting DNA lesions and chromosomal instability [34, 45]. Similar antioxidant-mediated protection has been reported previously, including reduced micronucleus formation and chromosome aberrations with vitamin C-vitamin E supplementation against 1,4-benzoquinone toxicity, and reduced benzene-induced micronuclei with quercetin treatment [43, 46]. In addition to reducing oxidative DNA damage, vitamin C may support genomic stability through its cofactor role for TET dioxygenases, which promote DNA demethylation and preserve hematopoietic cell integrity [25]. In our study, restoration of %PCE and PCE/NCE ratio toward normal values suggests recovery of marrow cellularity and proliferative capacity. This agrees with evidence that adequate ascorbate supports healthy hematopoietic stem cell differentiation and protects against defective differentiation and genomic instability in bone marrow [47, 48]. Thus, vitamin C appeared to protect the erythroid lineage from both the cytotoxic and genotoxic consequences of benzene exposure.

Benzene also caused marked leukopenia and distortion of leukocyte differentials, reflecting its immunosuppressive effect on bone marrow progenitors. In our benzene group, lymphocytes showed the most pronounced decline, while monocytes were also significantly reduced, in agreement with the known sensitivity of lymphoid cells to benzene toxicity [49, 50]. Previous work has shown that benzene preferentially suppresses T, B, and NK cell populations, producing lymphopenia and impaired adaptive immunity. In contrast, benzene-exposed animals in the present study showed a relative increase in neutrophils, along with eosinophilia and basophilia. This pattern may reflect a stress-related inflammatory response in which marrow injury and cytokine signaling stimulate granulocyte mobilization despite reduced lymphoid output. Cytokines such as interleukin-1, interleukin-8, and G-CSF may contribute to this

response [51, 52]. Similar mixed leukocyte changes have been described in exposed cohorts, where low-dose or subacute benzene exposure may cause relative neutrophilia together with lymphocyte depletion. Oxidative tissue injury and inflammatory signaling likely explain this imbalance.

Vitamin C co-treatment largely normalized the leukocyte profile. Total WBC counts recovered in vitamin C groups, suggesting protection against benzene-induced leukopoietic failure [34, 53]. Higher vitamin C doses were particularly effective in preserving lymphocyte and monocyte counts, thereby limiting immunosuppression. This effect may reflect the known role of vitamin C in supporting immune cell survival and function through reduced oxidative apoptosis and enhanced intracellular antioxidant systems such as glutathione [54, 55]. Vitamin C also appears to modulate chronic inflammatory signaling triggered by benzene by scavenging ROS and limiting NF- κ B-related cytokine production. Consistent with this mechanism, the benzene-induced increases in neutrophils, eosinophils, and basophils were significantly attenuated by vitamin C, indicating restoration of a more balanced myeloid output. Overall, vitamin C-treated rats showed a more physiological leukocyte distribution than untreated benzene-exposed animals, supporting its immunoprotective role in benzene-induced myelotoxicity and immunotoxicity [56, 57].

These leukocyte changes were further reflected in inflammatory cell ratios. In benzene-only rats, the NEU/LYM ratio was elevated, while the LYM/MON ratio was reduced, indicating systemic inflammation and immune dysregulation. The NEU/LYM ratio is a recognized marker of stress and inflammation, and its elevation reflects neutrophilia combined with lymphopenia, a pattern linked to chronic inflammation and poor prognosis in hematologic disorders [58]. The depressed LYM/MON ratio similarly suggests impaired adaptive immunity and marrow stress, which is consistent with reports of reduced lymphocytes and altered monocyte counts in benzene-exposed subjects [58]. Disturbance of the NEU/MON ratio further indicates that benzene shifted immune balance toward a pro-inflammatory, myeloid-skewed state while suppressing lymphoid elements.

Vitamin C corrected these abnormalities by shifting NEU/LYM, LYM/MON, and NEU/MON ratios toward normal ranges. By improving lymphocyte counts and reducing neutrophil predominance, ascorbic acid restored the balance between innate and adaptive immune compartments. This normalization is biologically meaningful because balanced leukocyte ratios reflect reduced inflammation and improved immunocompetence [54, 59]. These findings align with the established anti-inflammatory and immunomodulatory properties of vitamin C, including reduction of oxidative stress, limitation of inflammatory signaling, and stabilization of lymphocyte populations [60]. Vitamin C may also enhance monocyte and macrophage resistance to toxic stress, helping preserve monocyte numbers. The near-normalization of leukocyte indices in treated rats therefore indicates that vitamin C protected not only against quantitative immune cell loss but also against qualitative immune imbalance, thereby attenuating benzene-induced chronic inflammation and immune disequilibrium [61].

Benzene exposure also caused marked thrombocytopenia and altered platelet indices, indicating injury to the megakaryocytic lineage in a pro-inflammatory marrow environment. Platelet count and plateletcrit were significantly reduced in benzene-only rats, consistent with the myelosuppressive effect of benzene reported in animal studies and occupational exposure settings [62]. In addition, benzene-treated animals

showed reduced MPV and increased PDW, suggesting impaired platelet production and abnormal platelet size distribution. Low MPV in thrombocytopenic conditions may indicate defective megakaryocyte function, where the marrow fails to release the larger, younger platelets associated with active regeneration. Oxidative marrow injury caused by benzene likely contributes to impaired megakaryopoiesis and abnormal platelet morphology [63]. Platelet-related inflammatory indices were also affected. The PLT/LYM ratio increased because lymphocyte loss was proportionally greater than platelet loss, and elevated PLT/LYM is widely regarded as a marker of systemic inflammation and disease severity [64]. Likewise, SII was markedly increased, reflecting the combined effects of lymphopenia, relative neutrophilia, and platelet dysregulation.

Vitamin C improved these platelet disturbances in a dose-dependent manner. Platelet counts recovered toward control values, especially at higher doses, indicating protection against benzene-induced thrombocytopenia [39]. This restoration may be explained by antioxidant protection of megakaryocytes from oxidative apoptosis, support of their maturation, and preservation of the marrow microenvironment. Vitamin C is also involved in collagen synthesis and stromal maintenance, both of which are important for megakaryopoiesis. Its antioxidant effect may further help preserve the thrombopoietic milieu and reduce platelet injury caused by oxidative stress [65]. Plateletcrit was similarly restored, while normalization of MPV and PDW indicated more stable platelet production and morphology. Increased MPV from the abnormally low benzene values suggests release of newly formed larger platelets, whereas reduced PDW indicates a more uniform platelet size distribution. These findings are in line with previous reports showing that natural antioxidants can restore platelet counts and reduce platelet abnormalities in toxic injury models [36]. Vitamin C also lowered PLT/LYM and significantly reduced SII, indicating attenuation of neutrophil-driven inflammation, improved lymphocyte survival, and restoration of platelet homeostasis. Therefore, vitamin C not only preserved the thrombocytic lineage but also reduced the inflammatory consequences of thrombocytopenia, in agreement with studies showing improved redox status and lower inflammatory markers under toxic or metabolic stress [66].

One of the most striking findings in benzene-exposed rats was the high percentage of circulating blast cells, reaching 35% compared with 0% in controls. Because circulating myeloblasts are normally absent from peripheral blood, this marked increase strongly indicates leukemogenic progression. Benzene is a well-established leukemogen capable of inducing clonal proliferation of abnormal hematopoietic cells, and our findings suggest that even a 4-week exposure was sufficient to drive a pre-leukemic state toward overt blastemia. Mechanistically, benzene metabolites likely induced mutations and chromosomal lesions in hematopoietic stem and progenitor cells, thereby promoting clonal evolution and blast expansion [67, 68]. The combination of high blast burden and cytopenias in our model resembles an AML-like picture and emphasizes the severity of benzene-induced marrow injury.

Vitamin C co-treatment markedly suppressed this leukemic progression [69]. Peripheral blasts were dramatically reduced across all doses, reaching only 3–4% at 1000 mg/kg. This suggests that vitamin C interfered with critical steps in benzene-induced leukemogenesis, most likely by protecting hematopoietic stem cells from the initial DNA damage and chromosomal injury that trigger malignant transformation [70]. Vitamin C may also have promoted apoptosis or differentiation of abnormal cells that had already

emerged. Increasing evidence indicates that pharmacologic ascorbate can act as a selective pro-oxidant in malignant cells, promoting hydrogen peroxide generation and activating apoptotic pathways in leukemic blasts while sparing normal cells [1, 71]. In addition, vitamin C can reactivate silenced tumor-suppressor pathways through epigenetic mechanisms, particularly through restoration of TET2 and related hydroxylase activity, thereby promoting myeloid blast differentiation [72]. It has also shown synergy with epigenetic therapies in AML models and direct pro-apoptotic effects in acute lymphoblastic leukemia cells [73, 74]. Thus, vitamin C likely exerted a dual protective effect in our model - protecting normal hematopoietic cells from genotoxic transformation while also limiting survival or expansion of malignant clones. This resulted in a substantial reduction in blast burden and effective interruption of progression toward overt pre-leukemia. This finding is clinically relevant because circulating blast count is an important prognostic marker, and lower blast levels are generally associated with improved outcomes [74]. Our results also agree with previous studies in which natural antioxidants such as kolaviron reduced peripheral blasts and restored marrow morphology in benzene-induced leukemogenesis [36].

The anti-leukemic effect of vitamin C may also relate to its role in maintaining hematopoietic stem cell function. Recent evidence shows that ascorbate deficiency enhances HSC self-renewal and favors expansion of undifferentiated cells, thereby increasing the risk of clonal evolution [47]. By ensuring adequate ascorbate availability, vitamin C treatment may have supported more regulated HSC differentiation and limited abnormal blast expansion. Overall, these findings indicate that vitamin C acted as an effective chemopreventive agent in this benzene-induced pre-leukemia model. It reduced genotoxic injury, restored erythropoietic, immune, and platelet homeostasis, and markedly suppressed malignant blast proliferation. The near-normalization of blood counts, cytogenetic stability, and blast burden highlights a potent protective role of vitamin C in the hematopoietic system against benzene-induced pre-leukemic transformation. This is particularly important given the growing interest in high-dose vitamin C as an adjunct in cancer therapy, extending its relevance from treatment to prevention in toxic exposure settings [75]. In summary, vitamin C exerted multi-level protective effects - genotoxic, hematopoietic, immunologic, thrombopoietic, and anti-leukemic - against benzene toxicity and thereby helped prevent progression 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.

Declarations

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Conflicts of Interest: The authors declare no conflicts of interest.

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Figures

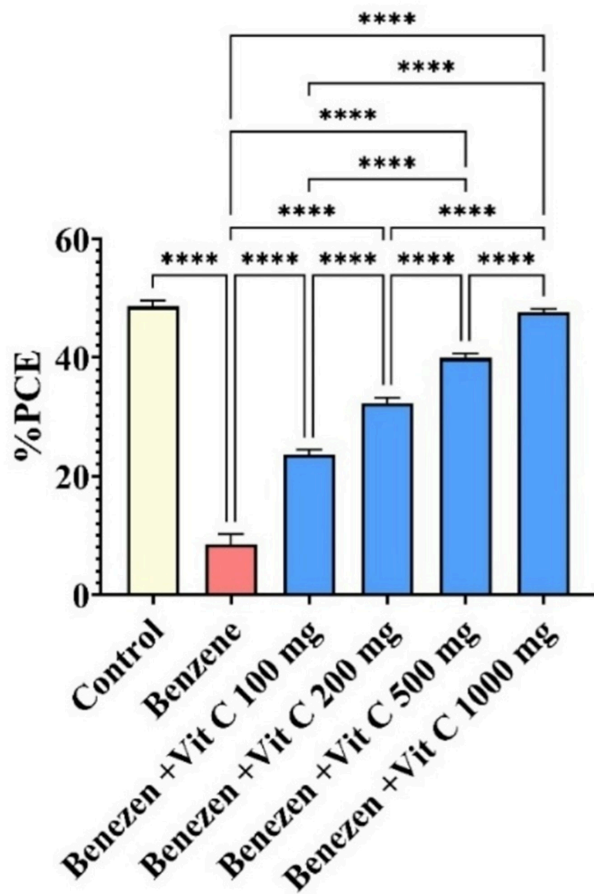
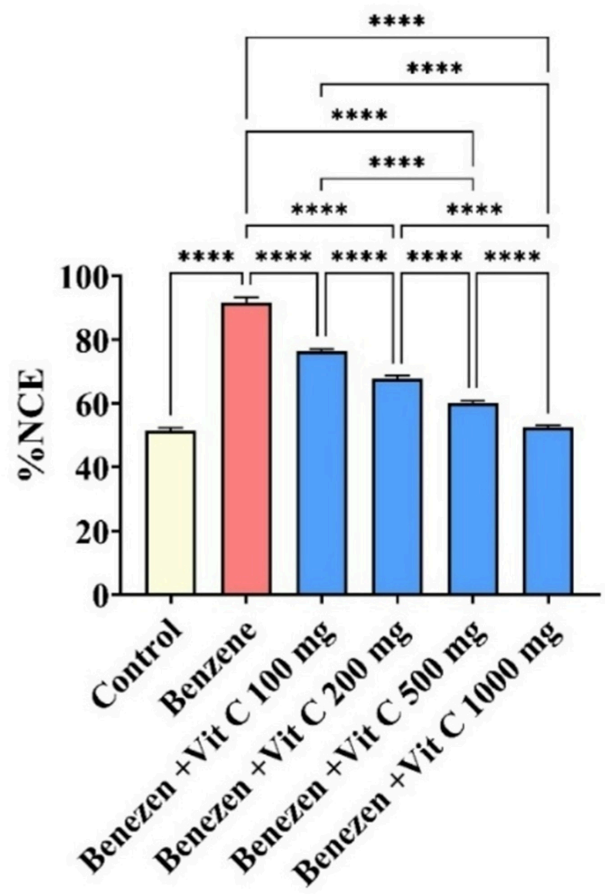


Figure 1

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

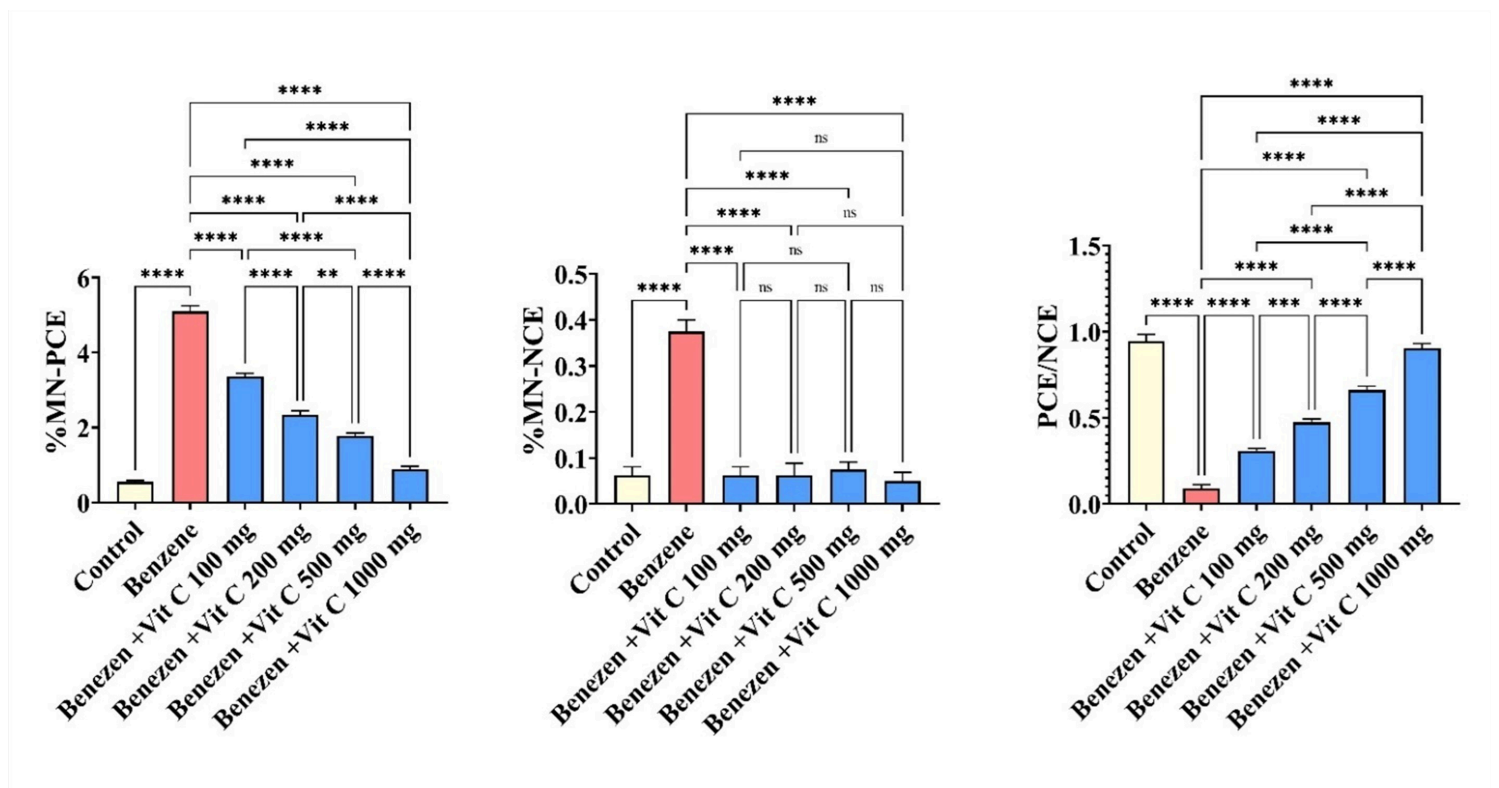


Figure 2

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

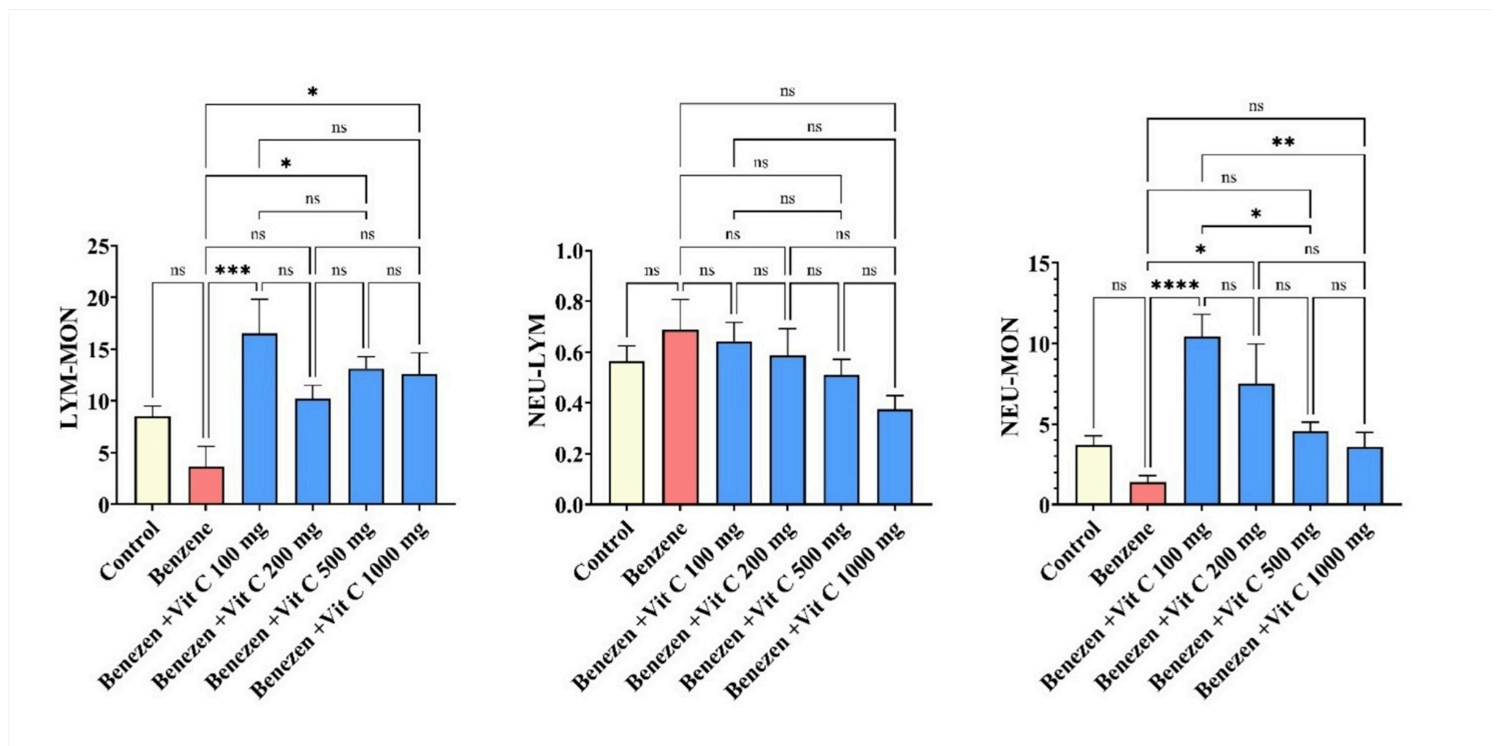


Figure 3

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

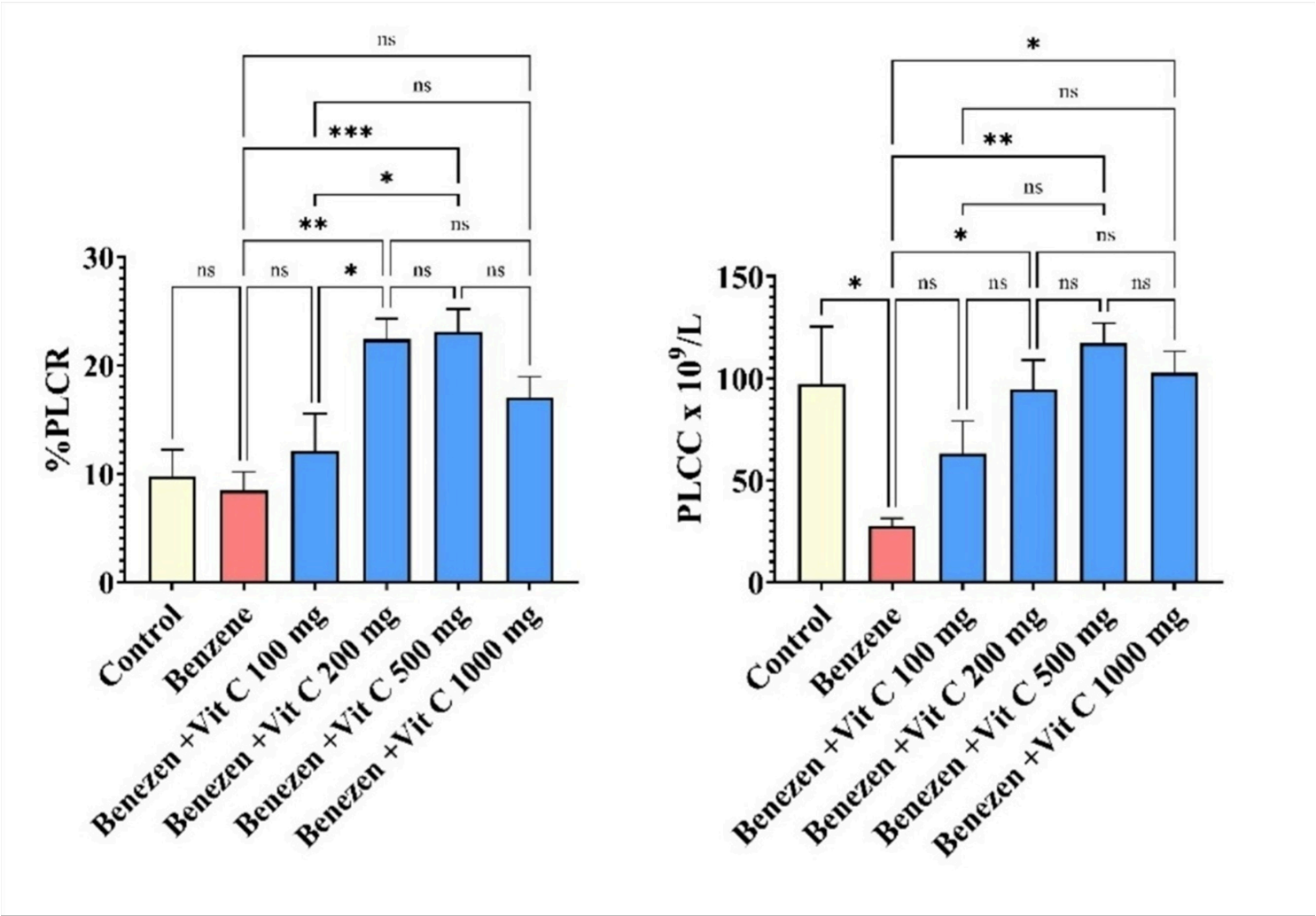


Figure 4

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

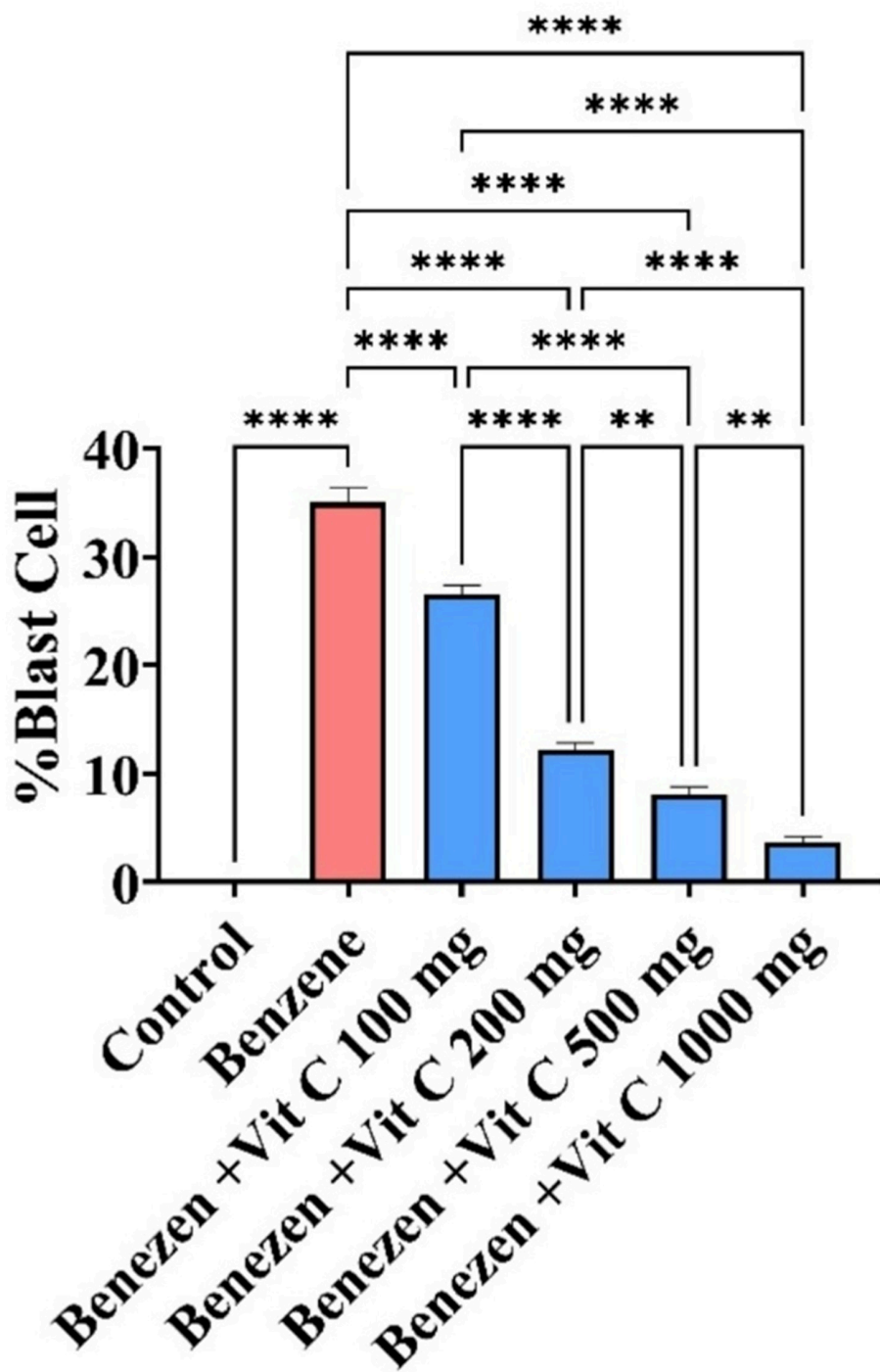


Figure 5

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

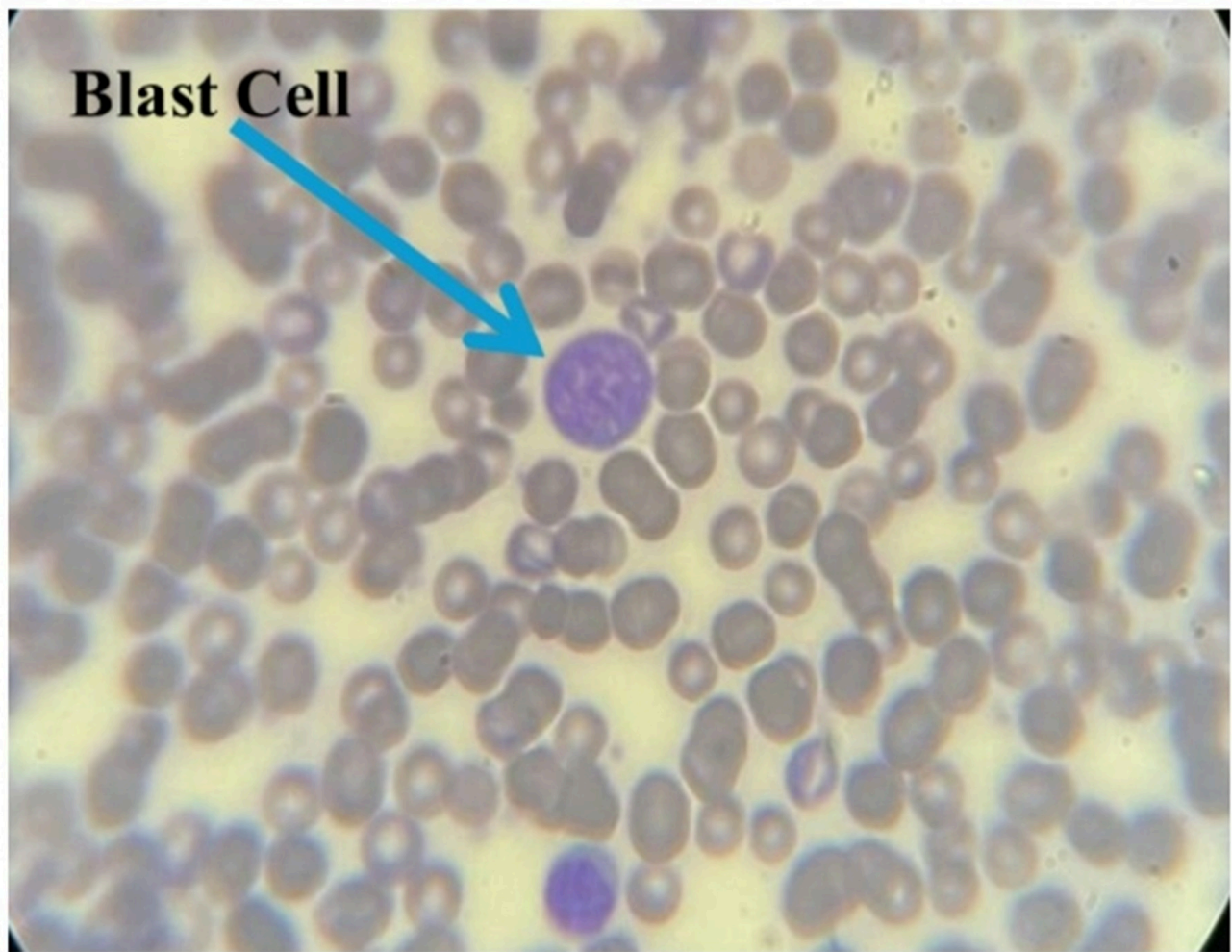


Figure 6

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