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Original Article

The influence of different doses of melatonin on Activin and Follistatin Gene expression levels against benzene-induced pre-leukemia in rats

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ABSTRACT

Background: Benzene exposure leads to early leukemogenic changes via oxidative stress and alterations in cytokine signaling in the marrow microenvironment. Melatonin has antioxidant and immunomodulatory action, but the regulatory effects of mediating the Activin A/Follistatin axis during benzene-induced pre-leukemia are still unclear. Materials and Methods: Rats were randomized to one of the experimental groups. The pre-leukemic model was established by repeated tail-vein administration of 0.2 mL benzene solution prepared in 2-propanol and distilled water (1:5:5, v/v) every 48 hours for a total duration of four weeks, or benzene plus oral melatonin (5, 10, 20, or 40 mg/kg/day for 4 weeks). Serum Activin A and Follistatin were measured by ELISA. Oxidative/antioxidant biomarkers 8-OHdG, MDA, SOD, and CAT were assessed by ELISA or TBA methods. Bone marrow Activin A and Follistatin transcripts were quantified by qPCR (ΔCt, 2^-ΔΔCt). Results: Benzene significantly reduced serum Activin A versus control and lowered CAT activity; Follistatin showed a modest, non-significant rise. MDA and 8-OHdG were lower than in the control after benzene. Melatonin produced dose-specific effects: (i) Activin A protein was not meaningfully restored at any dose; (ii) Follistatin peaked at 20 mg/kg; (iii) CAT improved at low-to-moderate doses, whereas SOD changes were not significant and MDA remained largely unchanged; (iv) 8-OHdG increased at 5-10 mg/kg but was comparatively lower at 20 mg/kg among melatonin groups. qPCR showed benzene downregulated Activin A and Follistatin transcripts versus control; melatonin 10 mg further reduced both relative to benzene. Conclusions: In benzene-exposed rats, melatonin modulated the Activin A/Follistatin pathway and selectively improved antioxidant defenses most consistently in CAT while further suppressing Activin A and Follistatin transcription, indicating a nuanced dose-dependent interaction without full normalization of Activin A protein levels.

Keywords: benzene-induced hematotoxicity; melatonin; activin A; follistatin; antioxidant; gene expression.

1 Introduction

Pre-leukemia is a class of hematological malignancies defined by clonal growth of abnormal blood cells, of which acute myeloid leukemia (AML) represents a particularly aggressive type [1]. AML results from the malignant transformation of myeloid progenitors in the marrow, resulting in fast proliferation of immature blasts and bone marrow failure. With new generations of therapies for the disease, AML is still challenging to treat and is often fatal, particularly for elderly individuals [2].These environmental and chemical exposures are established risk factors for pre-leukemia, and chronic exposure to benzene is a common industrial solvent and pollutant known to cause both human and animal aplastic anemia, myelodysplasia, and AML [3].

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Benzene's hematotoxicity derives from its bioactivation into reactive metabolites (e.g., benzoquinones), which damage hematopoietic stem cells and stromal cells and change cytokine signaling of the bone marrow microenvironment [3]. Benzene infusion induces the hypoplasia and leukemic transformation observed in AML in rats and, therefore, represents an established model for studying chemical leukemogenesis and chemoprotective interventions [4]. The oxidative pathways of leukemogenesis are closely associated with benzene-induced leukemogenesis. When the benzene metabolizing process produces reactive oxygen species (ROS), oxidative damage to deoxyribonucleic acid (DNA) and lipid peroxidation occurs in hematopoietic cells and may be responsible for a mutations and malignant transformation. 8-OHdG has long been recognized as a marker of oxidative DNA damage and correlated with augmented ROS-mediated genotoxic activity in benzene-exposed rats [5]. Malondialdehyde (MDA) is another significant indicator of oxidative damage and represents the peroxidation-related end

product of lipid damage which reflects injury to cellular membranes and is commonly elevated following benzene exposure [6]. Such conditions also disrupt the antioxidant defense system. In particular, superoxide dismutase (SOD) and catalase (CAT), which would normally detoxify superoxide radicals and hydrogen peroxide, may become suppressed or functionally impaired during benzene toxicity, thereby shifting the intracellular environment toward oxidative stress [7]. Activin A and Follistatin represent distinct biological entities with a distinction. Activin A belongs to the TGF-β superfamily and is associated with cellular differentiation and immune control, while Follistatin binds to and neutralizes Activin A through which it modulates its bioavailability. From recent clinical studies, Activin A has been reported to be upregulated in AML patients at the time of diagnosis and downregulated following remission so that it represents a factor in the leukemic bone marrow niche or disease activity [9, 10]. In contrast, Follistatin is upregulated in AML and could support leukemogenesis by sequestering Activin A tumor-suppressing signals [11]. High-risk subset Follistatin overexpression was identified as a potential therapeutic target in these studies: dysregulated Activin A/Follistatin axis can modulate pre-leukemia cell proliferation and survival in FLT3-ITD mutant AML [12]. Yet very little is known about how these genes function in toxin-induced leukemic models. So far, there are no published studies to quantify in vivo the activation of Activin A and Follistatin genes in benzene-caused AML. It would be useful to investigate their expression in a vivo model to determine whether the influence of benzene on leukemogenesis involves not only the disruption of growth factor signaling, but also oxidative damage. Given the complexity of cytokine signaling along with oxidative stress in pre-leukemia, there is a developing interest in drugs effective in modulating both pathways. Melatonin as a neurohormone synthesized in the pineal gland has proved to be an attractive candidate owing to its complex biological actions. Melatonin also possesses strong antioxidant and free radical scavenging effects, by directly neutralizing ROS and upregulating antioxidant enzymes and protecting cells from oxidative damage [13, 14]. It has additional oncostatic effects on hematological cancer: melatonin induces apoptosis and inhibits proliferation of pre-leukemia cells, and there is synergy in cytotoxic effects in combination with chemotherapy [15]. Melatonin has several anticancer effects (beyond antioxidant effect), and not only can it change gene expression and signaling pathways that help control cell survival and generate immunodefensive responses. Previous in vitro studies showed that melatonin was sufficient to induce cell death in FLT3-ITD-positive AML cell lines and to inhibit their growth in vivo and thus presented promising therapeutic applications in aggressive pre-leukemia subtypes [16, 17]. Melatonin also exerts cytoprotective function against common hematopoietic cells and the bone marrow microenvironment and it might reduce the collateral damage of oxidative insult [18]. These are signals that melatonin may counteract benzene-induced leukemic pathology by both mitigating oxidative molecular damage and regulating aberrant cytokine dynamics. Although natural compounds and antioxidants have been shown to have antioxidant roles against benzene-induced leukemia [19, 20], few relevant data have been made available relating to changes in specific gene expression pathways of various natural compounds and antioxidants in pre-leukemia. Certainly, there's an unknown profile for Activin A and Follistatin in the context of chemical-induced AML or regulation of their expression by melatonin. The interactions of markers of oxidative stress with Activin/Follistatin signaling in benzene-induced leukemia have been unexplored representing a significant knowledge gap. Bridging this gap might shed new light on some of the aspects of leukemogenesis and provide new molecular targets for therapy [21, 22]. The purpose of the current study was to assess how melatonin may impact on the gene expression of Activin A and Follistatin, oxidative stress and antioxidant markers, in a rat model of induced-benzene AML. By assessing molecular signaling through the Activin/Follistatin axis, oxidative injury markers (MDA and 8-OHdG), and antioxidant defense markers (SOD and CAT), this study aimed to determine how melatonin-mediated effects may alleviate benzene-induced leukemic changes.

2 Materials and Methods

2.1 Animals

Rats aged 8-10 weeks, weighing 180-220 g were used for the studies. Animals were kept under standard laboratory conditions during 12-hour light/dark cycles with ad libitum access to food and water. All procedures were carried out according to animal care and guidelines and the study protocol was approved by the Institutional Animal Ethics Committee of Cihan University-Erbil, Approval Number (CUE-REC/2025/ 09).

2. 2 Chemical Reagents and Preparation

A benzene solution was prepared by adding benzene (Chem Lab, Belgium), 2-propanol, and distilled water in a 1:5:5 volume ratio (benzene:2-propanol: distilled water, v/v). Volumetrically measured all components were transferred to a sealed container and mixed thoroughly until homogeneity could be achieved. Melatonin powder (Bioven Ingredients, India) was weighed accurately on an electronic balance and dissolved in distilled water, creating a stock solution, to be diluted for making 4 dosing preparations equal to 5, 10, 20, and 40 mg/kg body weight. Each preparation was vortex-mixed for homogeneity and individual dose volumes were calculated for each animal according to its current body mass.

2. 3 Protocol for Inducing Pre-leukemia with Benzene

Pre-leukemia in rats was induced by repeated intravenous injection of a benzene solution. A solvent system consisting of benzene, propanol, and distilled water at a 1:5:5 (v/v) ratio was used. Each rat was administered 0.2 mL via the tail vein every 48 hours for four consecutive weeks. This dosing sequence was selected to permit both systemic and early genotoxic assessment preceding pre-leukemic transformation. Throughout the whole experiment, animals were systematically followed for physiological and behavioral changes and were observed before and after each injection as well as during the course of the

experiment. The induction method and dosing pattern are according to the method presented in [3].

2. 4 Experimental Design and Group Assignments

Rats were randomly divided into six groups to assess treatment effects during benzene-induced pre-leukemia. The control group (G1) was given oral distilled water daily for 4 weeks. The benzene pre-leukemia group (G2) followed the induction procedures outlined in Section 2.3 where they received 0.2 mL per infusion of the benzene:2-propanol: distilled water solution (1:5:5, v/v) through the tail vein every 48 hours for 4 weeks without other active treatment. Groups G3-G6 received the same benzene induction as G2, and also received once-daily oral melatonin for four consecutive weeks with gradually increasing doses based on body weight: 5 mg/kg (G3), 10 mg/kg (G4), 20 mg/kg (G5), and 40 mg/kg (G6). The dose volumes were tailored to the present mass of each rat at the time of administration. Oral preparations were freshly made in distilled water and administered at around the same time each day in order to minimize variability. Animals in all conditions were observed during the study in order to confirm dosing, to assess tolerance and adherence with the scheduled treatment. The study design included a vehicle-treated control group, a benzene-induced pre-leukemia comparator group, and four melatonin treatment groups administered from low to high doses, thus allowing possible dose-dependent effects on our studied outcomes to be evaluated.

2. 5 Antioxidants and Free Radicals Biomarkers

The SOD, CAT, and 8-OHdG were all quantified by the identical technology as in Sunlog Biotech ELISA kits and all tests were conducted according to strict manufacturers' regulations. Samples, standards, and controls were assayed in duplicate, as described and all measurements were recorded for the reliability". MDA as well as other measures of lipid peroxidation were measured with the thiobarbituric acid (TBA) assay. In brief, 150 µL of serum was deproteinized with trichloroacetic acid (TCA) and incubated for 45 min at 95 °C; at this point, tubes were cooled down as needed, centrifuged to clarify the supernatant and the absorbance of reaction product was read spectrophotometrically at 532 nm against a suitable blank. The following formula serves for MDA concentrations (µmol/L): MDA (µmol/L) = $A_{532} \times D \times L \times E_0$; A_{532} is absorbance at 532 nm, D is dilution factor, L is optical path length and E_0 is extinction coefficient. Spectrophotometric parameters (such as wavelengths) were modified accordingly and as per methodological recommendations for this and other biochemical assays. Serum Activin-A and Follistatin were analyzed according to manufacturers' specifications without deviation by sandwich ELISA (Sunlog Biotech): serum was diluted according to specifications, standards were prepared simultaneously, samples were assayed in duplicate on capture-antibody coated plates, followed by consecutive incubations with biotinylated detection antibodies and Horseradish Peroxidase conjugate, supplemented with Tetramethylbenzidine substrate, reactions stopped, and absorbance quantified at 450 nm. Concentrations of analytes were calculated from the interpolating four-parameter logistic (4-PL) standard curve of the kit standards [14].

2. 6 Quantitative Gene Expression Profiling of Activin A and Follistatin

Total RNA was obtained from peripheral blood and bone marrow following the manufacturer's protocol using the QIAamp RNA Blood Mini Kit. The RNA was then reverse-transcribed to cDNA using the UltraScript kit. Quantitative PCR (qPCR) was performed with gene-specific primers for Activin A and Follistatin using identical cycling conditions for all reactions. Expression data were normalized to the housekeeping gene, β-actin. Relative transcript abundance was determined by the 2^-ΔΔCt method, expressed as fold change relative to the calibrator group [15]. Reagent preparation and amplification conditions were standardized for all runs for analytical reliability, and each assay was performed in duplicate.

2. 7 Statistical Analysis

Data were analyzed by one-way ANOVA followed by Tukey's post hoc test for multiple pairwise comparisons. Results are shown as mean ± SEM. Assumptions of normality and homogeneity of variances were evaluated before performing inferential tests. Bar charts were generated, and analyses were performed using GraphPad Prism (version 9.0.0). Statistical significance was set at $p < 0.05$.

3 Results

3. 1 Antioxidant and Hormonal Biomarkers

Figure 1 shows that benzene caused a small but non-significant increase in follistatin from 85 ng/ml in controls to 100 ng/ml. Melatonin at 5 mg and 10 mg yielded 88 ng/ml and 95 ng/ml, respectively, with no significant change versus benzene, whereas the 20 mg dose reached 140 ng/ml, significantly higher than benzene ($p < 0.001$) and control ($p < 0.01$) and also higher than the 40 mg dose (82 ng/ml; $p < 0.0001$), indicating a peak effect at 20 mg. Activin A decreased significantly after benzene exposure from 180 pg/ml in controls to 100 pg/ml ($p < 0.01$), and none of the melatonin doses restored it meaningfully (5 mg = 112 pg/ml; 10 mg = 122 pg/ml; 20 mg = 115 pg/ml; 40 mg = 105 pg/ml; all nonsignificant vs benzene).

Figure 2 indicates that the DNA oxidation marker 8-OHdG measured 40 ng/L in controls and 25 ng/L in benzene, then increased with melatonin at 5 mg to 60 ng/L, at 10 mg to 70 ng/L, and at 40 mg to 45 ng/L (p values ranging from $p < 0.05$ to $p < 0.001$ vs benzene), while 20 mg produced a lower value of 35 ng/L relative to the other melatonin doses. SOD remained unaltered across groups (control 3.5 pg/L; benzene 2.5 pg/L; melatonin 5, 10, 20, and 40 mg at 3.0, 3.2, 3.1, and 2.8 pg/L; all comparisons nonsignificant). MDA decreased from 15 µmol/L in controls to 10 µmol/L after benzene ($p < 0.001$) and showed no further change with melatonin (5 mg = 10.5 µmol/L; 10 mg = 10.0 µmol/L; 20 mg = 9.5 µmol/L; 40 mg = 10.0 µmol/L; all

nonsignificant vs benzene). CAT dropped from 25 pg/ml in controls to 15 pg/ml in benzene ($p < 0.01$); melatonin partially restored CAT at 5 mg to 22 pg/ml ($p < 0.001$ vs benzene), at 10 mg to 20 pg/ml ($p < 0.01$), and at 20 mg to 18 pg/ml ($p < 0.05$), whereas 40 mg remained 15 pg/ml with no improvement. Overall, melatonin at 20 mg/kg produced the most favorable biochemical profile, maximizing follistatin at 140 ng/ml, minimizing 8-OHdG at 35 ng/L among melatonin doses, and improving catalase to 18 pg/ml, while Activin A suppression persisted around 100-122 pg/ml across all melatonin groups, and higher or lower melatonin doses were less effective or neutral on the measured endpoints.

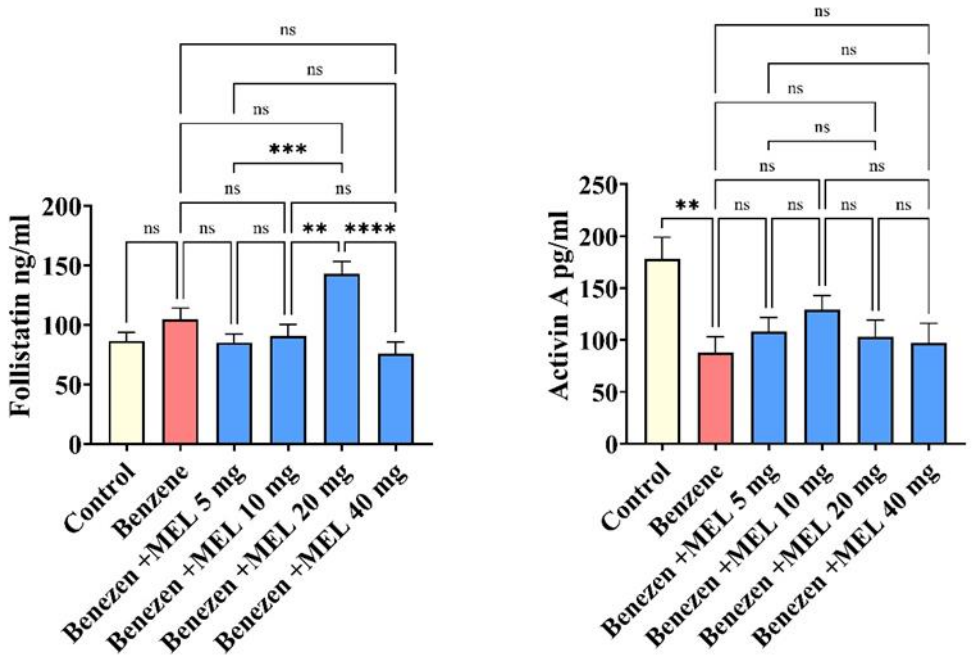
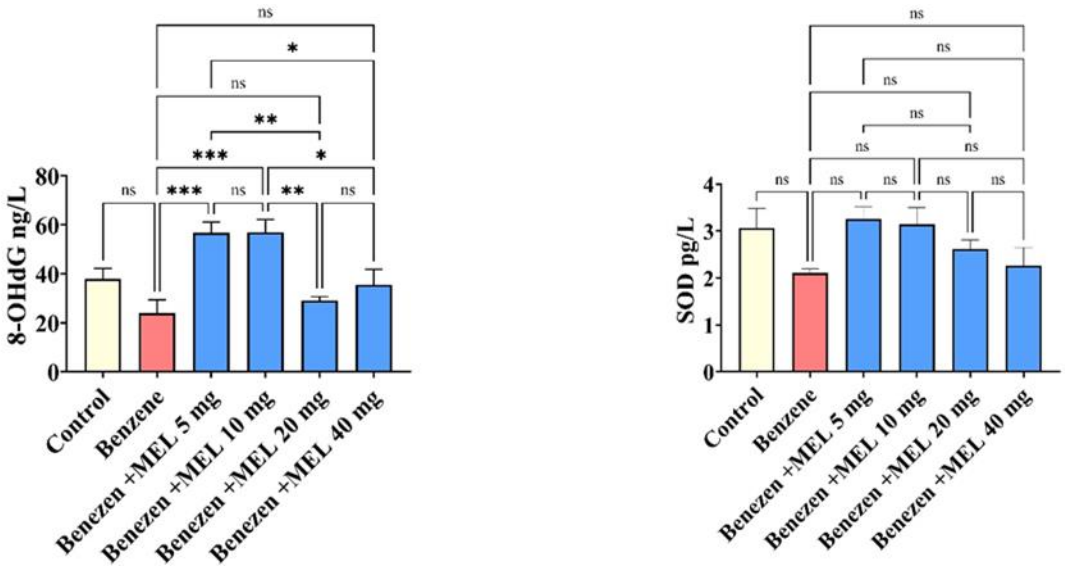


Figure 1: Effects of graded melatonin dosing (5-40 mg/kg) on serum follistatin and activin A levels in benzene-exposed rats.



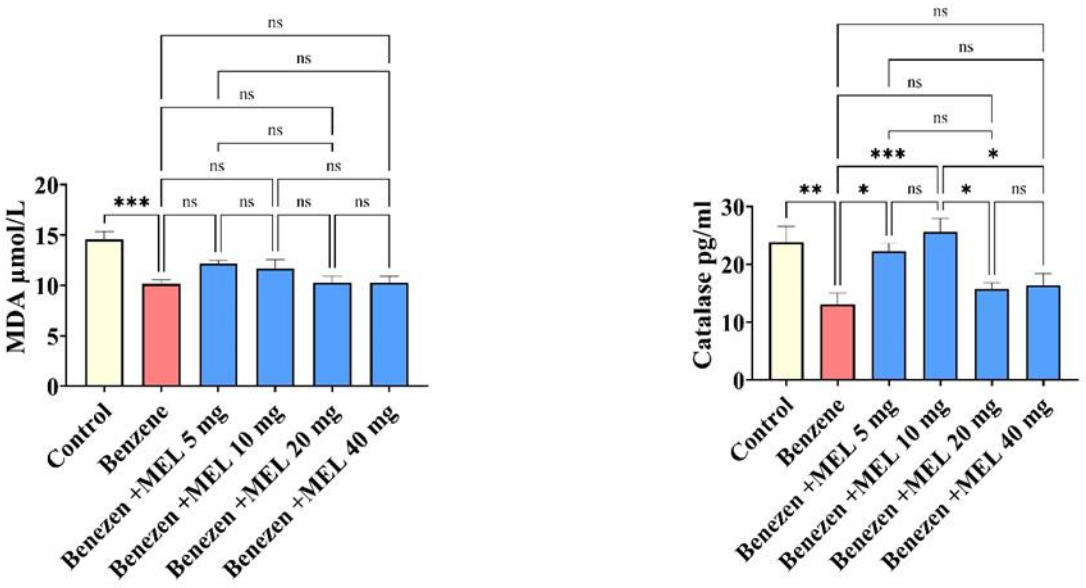


Figure 2: Effects of graded melatonin dosing (5-40 mg/kg) on oxidative stress markers in rats with benzene-induced pre-leukemia (8-OHdG, MDA, SOD, catalase).

Table 1: Effects of different doses of melatonin on serum Activin A, Follistatin, oxidative stress, and antioxidant biomarkers in benzene-induced pre-leukemic rats.

Group	Activin A	Follistatin	8-OHdG	SOD	CAT	MDA
Control	178.1 ± 20.84	86.46 ± 7.113	37.86 ± 4.336	3.061 ± 0.4201	23.82 ± 2.771	14.56± 0.8115
Benzene	87.90 ± 15.29**	104.5 ± 9.268	23.91 ± 5.489	2.104 ± 0.09116	13.12 ± 1.887**	10.15±0.3905 ***
Benzene +MEL 5 mg	108.3 ± 13.37	85.01 ± 7.414	56.71 ± 4.376***#	3.259 ± 0.2555	22.29 ± 1.334*	12.18±0.3337
Benzene+ 10 mg	129.3 ± 13.62	90.62± 9.364	56.93 ± 5.248***#	3.145 ± 0.3605	25.66 ± 2.326***#	11.70±0.8377
Benzene +MEL 20 mg	102.7 ± 16.24	142.4± 10.43###	29.08 ± 1.583	2.615 ± 0.1963	15.74 ± 1.071	10.28±0.6702
Benzene +MEL 40 mg	97.39 ± 18.74	75.50 ± 10.02	35.57 ± 6.243	2.259 ± 0.3928	16.43 ± 2.027	10.28±0.6670

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

Activin A, Follistatin, 8- OHdG: 8-hydroxy-2'-deoxyguanosine, SOD: Superoxide dismutase, CAT: Catalase, MDA: Malondialdehyde

3. 2 Gene Expression Analysis of Activin A and Follistatin Transcripts

Figure 3 illustrates ΔCt values for Activin A and Follistatin across the control, benzene, and benzene + melatonin (10 mg/kg) groups. For Follistatin, Control measured -1.0, Benzene increased to 1.0 ($p < 0.05$ vs Control), and Benzene + MEL 10 mg rose further to 1.4 (ns vs Benzene), indicating that benzene increased the ΔCt value for Follistatin, reflecting reduced gene expression, while melatonin (10 mg/kg) slightly increased ΔCt further without statistical significance. For Activin A, Control was -0.4, Benzene was 0.8 (ns vs Control), and Benzene + MEL 10 mg was 2.0 (ns vs Benzene), showing a higher ΔCt value after melatonin treatment, which corresponds to lower relative gene expression but without statistical significance compared with the benzene group. Overall, benzene significantly alters Follistatin transcription, whereas melatonin (10 mg/kg) did not produce a statistically significant additional effect on Activin A expression at the ΔCt level.

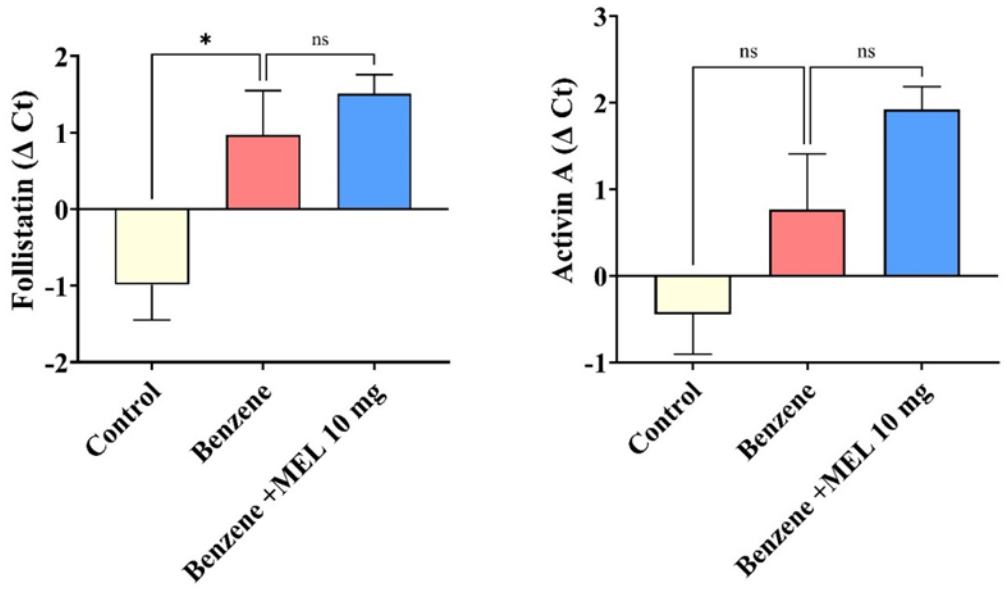


Figure 3: Comparison of ΔCt values for Activin A and Follistatin gene expression in experimental groups in Benzene-Induced pre-leukemia rats.

3. 3 Fold-Change Analysis of Activin A and Follistatin Gene Expression

Table 2 summarizes the relative expression of Activin A and Follistatin after benzene exposure and is derived with respect to the control group as this experimental baseline. With respect to Activin A, ΔCt changes from -0.4475 in controls to 0.7656 in benzene to give a ΔΔCt of 1.2131. This translates to a 0.43-fold change with an approximate 57% decrease in Activin A gene expression after benzene treatment. Follistatin has the more pronounced implication: ΔCt increases from -0.9863 in controls to 0.9693 in benzene, giving a ΔΔCt of 1.9556. As a result, the fold change is 0.26, which is more than 74% reduction in Follistatin gene expression. Overall, the higher ΔΔCt value and lower fold change seen with Follistatin indicate that the gene might be more sensitive to benzene-induced transcriptional reduction than Activin A, since both genes are decidedly downregulated relative to their control baseline.

Table 2: Relative fold change in Activin A and Follistatin gene expression in benzene-treated rats compared with the control group.

Group	ΔCt Mean (Activin A)	ΔΔCt (vs Control)	Fold Change (2^-ΔΔCt)	ΔCt Mean (Follistatin)	ΔΔCt (vs Control)	Fold Change (2^-ΔΔCt)
Control (Reference)	-0.4475	0.0	1.000 (reference)	-0.9863	0.0	1.000 (reference)
Benzene	0.7656	1.2131	0.43× ↓	0.9693	1.9556	0.26× ↓↓

3. 4 Fold-Change Analysis of Activin A and Follistatin Expression in Melatonin-Treated Groups

Table 3 presents the quantitative PCR analysis of Activin A and Follistatin gene expression in the benzene-only group compared with the benzene + melatonin (10 mg/kg) group. which is represented as ΔCt means and ΔΔCt values, and the fold changes from the 2^-ΔΔCt assay method. The mean ΔCt value for Activin A was 0.7656 in the benzene group and 1.924 in the benzene + melatonin (10 mg/kg) group indicative of a higher threshold cycle and smaller gene expression for Activin A. Based on the calculation of ΔΔCt results, when we compare Activin A between MEL-treated and benzene groups 1.1584, we can see a fold change of 0.44× or a 56% decrease in gene expression of MEL-treated rats compared to that of benzene alone. This reduction suggests that melatonin (10 mg/kg) may further suppress Activin A gene expression compared with benzene exposure alone but such an association wasn't statistically significant in the preceding figure. For Follistatin, the mean ΔCt value in the benzene group was 0.9693 whereas ΔCt of the MEL 10 mg group was 1.508, also showing lower transcript levels in the treated group. The ΔΔCt value of 0.5387 corresponds to a fold change of 0.69, indicating an approximate 31% reduction in Follistatin gene expression following melatonin treatment. After melatonin treatment relative to benzene treatment only. Although the previous bar graph presented an upward slope of ΔCt with MEL treatment, the relative fold change analysis in this study demonstrates that melatonin (10 mg) also lowered the relative expression of both Activin A and Follistatin genes, when compared with benzene as the reference group. Overall, fold-change analysis (2^-ΔΔCt) demonstrates that benzene downregulated both Activin A and Follistatin relative to the control group, while melatonin (10 mg/kg) further reduced their transcript levels compared with the benzene group.

Table 3: Fold Change in Gene Expression Relative to Benzene Group (Activin A) and (Follistatin).

Group	ΔCt Mean (Activin A)	ΔΔCt (vs Benzene)	Fold Change (2^-ΔΔCt)	ΔCt Mean (Follistatin)	ΔΔCt (vs Benzene)	Fold Change (2^-ΔΔCt)
Benzene	0.7656	0.0	1.000	0.9693	0.0	1.000 (reference)
Benzene + MEL 10 mg	1.924	1.1584	0.44× ↓	1.508	0.5387	0.69× ↓

4 Discussion

Benzene exposure, however, disrupted oxidative balance in this study, changed cytokine regulation in the bone marrow microenvironment, and the melatonin treatment had only a partial but measurable protective effect. Benzene treatment was shown to be associated with decreased CAT activity, decreased MDA and lower 8-OHdG in this experimental model compared to control group while SOD was not significant. Melatonin exhibited dose-dependent effects: CAT was improved at 5-20 mg/kg, particularly the 5-10 mg/kg group, SOD values were not altered, MDA did not differ significantly from benzene and 8-OHdG levels were higher at 5-10 mg/kg but lower at 20 mg/kg relative to the other melatonin treatment groups. Overall, these results indicate that melatonin partially resurrected antioxidant response to be seen most obviously in CAT activity, in the absence of uniform normalization (by its endpoints) of all oxidative signature. Existing studies repeatedly associate benzene exposure to oxidative stress and DNA damage in hematopoietic tissues. In addition, chronic benzene exposure in rats has been found to raise 8-OHdG and also to induce lipid peroxidation, again confirming its ability to induce oxidative lesions in hematopoietic bodies [5]. Similarly, oxidative insult by benzene has been linked to DNA mutagenesis and pre-leukemic transformation [18]. Previous studies also showed that the oxidative stress mediated MDA along with antioxidant enzymes in blood and bone marrow was increased and suppressed after benzene exposure, revealing a highly deleterious oxidative imbalance [19]. Recent toxicological data also indicated an increased MDA and decrease in SOD and glutathione in benzene-exposed rats and supported benzene's ability to shift the redox state toward oxidative-dominated environment. In such a context, melatonin seems to suppress a certain amount of the oxidative damage induced by benzene. Its beneficial effects in our study were primarily observed in the enhancement of CAT activity, however, in SOD, MDA and 8-OHdG in 5-10 doses also were more varied. Nonetheless, melatonin is recognized in present literature as a highly effective free radical scavenger and antioxidant enzyme activity regulator and an antioxidant-suppressive endogenous antioxidant defense system enhancer [9]. Its antioxidant activity has been associated with direct scavenging of reactive oxygen species (ROS) and upregulation of antioxidant enzymes, in part activated via the Nrf2/ARE pathway [23]. In this study's rats treated with melatonin, the relative improvement is clear evidence of both direct neutralization of free radicals and support for enzymatic antioxidant defenses. The results were consistent with earlier studies reporting antioxidant-based interventions attenuating benzene-induced oxidative damage. For example, linalool has been found to reduce hepatic MDA levels and enhance antioxidant status in benzene-exposed rats [24]. In an analogous manner, Nigella sativa extract reduced benzene-associated accumulation of 8-OHdG and attenuated hematological changes [25]. Atriplex halimus extract can also potentiate antioxidant defense via the increase in CAT activity and glutathione under benzene-induced toxic events [26]. Collectively these findings lend support to the idea that augmenting antioxidant defense mechanisms could offset at least some of the pro-oxidant activity of benzene in blood and hematopoietic tissues. In the current investigation, melatonin seemed to also be associated with a dose related protective influence, because increasing concentrations of melatonin generally predicted more normalised data. This pattern is consistent with the dose-dependent response earlier characterized in experimental models of toxic exposure [21]. Since oxidative DNA damage such as 8-OHdG formation is implicated in mutagenesis and chromosomal instability responsible for AML initiation suppression of such lesions may be one of the most important mechanisms whereby melatonin inhibits benzene-facilitated leukemogenic changes [27]. In other words, the relatively small degree of change in oxidative balance could be biologically informative in attenuating bone marrow damage and delaying the transition toward malignant transformation. Apart from oxidative stress, the present results also indicate that benzene disrupts the activation of the Activin A/Follistatin signaling pathway, and therefore its pathogenic action could be related with dysregulation in the cytokine- (and growth factor) mediating signaling in the bone marrow microenvironment. In benzene-treated rats, serum concentrations of Activin A and Follistatin, together with their marrow gene expression, were altered compared with controls. This is a notable observation because most previous benzene studies have focused on hematological, oxidative, or genotoxic outcomes rather than this specific cytokine pathway. Our data therefore suggest, for the first time in this model, that benzene-induced pre-leukemic injury may involve disruption of Activin A/Follistatin signaling [28].

Biological speculation of this kind is supported by data from AML studies. Activin A belongs to the TGF-β superfamily, which is highly involved in cell proliferation, differentiation, and inflammatory regulation. It has been demonstrated that AML pathogenesis and disease progression can be directly related to disruption of the Activin A/Follistatin balance, with Follistatin acting as the endogenous antagonist binding to and neutralizing Activin A [24]. High Activin A levels have also been linked to an increase in leukemia burden and disease activity [25], whereas Follistatin overexpression is associated with more aggressive AML phenotypes and could, in turn, contribute to the survival of leukemic cells by inhibiting the growth-inhibitory effects of Activin A [26]. Here, the altered Activin A and Follistatin expression in the benzene-only group indicates the activation of

an adverse signaling cascade mimetic of clinical leukemia. Elevated Activin A indicates a reactive mechanism in response to cellular injury, inflammation, and tissue remodeling, while higher levels of Follistatin might act as either a compensatory mechanism or a maladaptive process to retain abnormal cell survival by inhibiting tumor-suppressive signaling [27]. Together, these changes should promote expansion of malignant clones by developing a bone marrow microenvironment that is more permissive to leukemic progression [28].

Crucially, melatonin treatment also modulated this dysregulated cytokine release. Melatonin-treated groups, particularly at higher doses, showed lower Activin A and Follistatin levels than the benzene group, together with downregulation of their gene expression. It is inferred that melatonin may not only function as an antioxidant, but also assist in re-establishing cytokine homeostasis in the marrow microenvironment. The effect is important since it suggests that melatonin might impact upstream mechanisms of disease in addition to neutralizing oxidative products.

Several mechanisms may explain this action. First, by reducing oxidative and genotoxic stress, melatonin may decrease the injury-driven stimulation of Activin A and associated signaling in stromal or immune cells. Activin A is commonly induced in tissues undergoing oxidative damage, inflammation, or repair, so the antioxidant action of melatonin may indirectly normalize its expression [29]. Second, melatonin is known to possess direct immunomodulatory properties. It suppresses pro-inflammatory transcription factors such as NF-κB and modifies cytokine-related gene expression [9]. It has also been reported to interfere with upstream inflammatory and redox-sensitive pathways, including sirtuin-1 and Nrf2-linked mechanisms, thereby limiting cytokine overproduction [18]. Moreover, melatonin has been shown to influence TGF-β family signaling in several disease models, including fibrotic and inflammatory conditions [30]. In this study, these combined actions may have contributed to the restoration of a more balanced Activin A/Follistatin milieu in bone marrow.

The reduction of Follistatin in particular may be relevant for leukemia control. Because Follistatin antagonizes Activin A, its suppression could remove an important brake on Activin-mediated growth-inhibitory or differentiation-supporting signals [31]. Although Activin A itself also declined toward normal values in melatonin-treated animals, this concurrent normalization of both ligand and antagonist likely reflects resolution of the upstream injury and inflammatory stimuli driving their dysregulation. In other words, melatonin seems not simply to stimulate or suppress one component of the pathway, but rather to reduce the abnormal activation of the entire axis caused by benzene exposure [32].

These results are in line with that oxidative stress and cytokine disturbance are mechanistically intertwined in benzene-induced pre-leukemia. Benzene metabolites produce reactive oxygen species that damage DNA and membrane lipids in bone marrow cells while simultaneously activating inflammatory and growth-regulatory pathways that may contribute to malignant transformation [33]. In the current benzene-only rat model, the association was evident with clear oxidative alterations accompanied by disruption of the Activin A/Follistatin signaling axis. Melatonin might inhibit this pathogenetic cascade at several levels: free radical scavenging, enhanced antioxidant defense, reducing DNA damage, and blockade of ROS-responsive inflammatory signal transduction (e.g., NF-κB and MAPK). Together, melatonin could suppress the excessive release of cytokines and growth factors from the bone marrow niche, and therefore create a less conducive setting for leukemic development. This interpretation is consistent with the growing literature related to the antileukemic effects of melatonin. In AML-based experimental systems, melatonin indicated cytotoxic and growth-inhibitory activity especially in aggressive FLT3-ITD [10, 11]. The underlying mechanisms of action were suggested to be perturbation of cancer metabolism, oxidative stress pathways, and signaling networks that contribute to cell survival. Melatonin was also found to induce apoptosis in malignant cells with the modulation of Bcl-2 family proteins and caspases [34]. Additionally, our in vivo observations suggest that melatonin can protect the normal marrow from oxidative damage by making the abnormal pre-leukemic cells less able to proliferate or survive. While our analysis did not directly evaluate marrow cellularity, blast burden, or apoptosis markers, the normalization of oxidative and cytokine parameters suggests a marrow microenvironment that is not as conducive to leukemogenesis. In addition, the beneficial effects of melatonin were increased with dose, with the larger doses reaching near-normalization of various measured parameters. It is possible that melatonin exists in a therapeutic window during which antioxidant, anti-inflammatory, and signaling-modulatory activities are optimized. At the same time, some improvement was still evident even at lower doses, indicating that biologically relevant protection may occur within tolerable dose ranges. Given the known safety profile of melatonin and its availability in bone marrow tissues, these findings may have translational significance [35, 36].

Overall, the present findings support melatonin as a multifunctional chemoprotective agent in benzene-induced pre-leukemic conditions. Its actions appear to involve both attenuation of oxidative damage and correction of disrupted cytokine signaling. These two processes are likely interdependent and together may help protect the hematopoietic niche from the cumulative molecular events that promote leukemic transformation. Thus, melatonin may hinder benzene-related leukemogenesis by restoring redox homeostasis, limiting oxidative DNA injury, and re-establishing a more balanced Activin A/Follistatin axis.

In summary, this discussion provides evidence that melatonin exerts a multi-target protective effect against benzene-induced pre-leukemic changes in vivo. By modulating oxidative stress and cytokine signaling simultaneously, melatonin may interfere with two major pathogenic pathways involved in chemical leukemogenesis. These findings strengthen the mechanistic rationale for considering melatonin as a potential chemoprotective candidate for further investigation in

environmentally induced pre-leukemic models and support the broader concept that targeting both redox biology and growth factor signaling may be an effective strategy against benzene-associated marrow injury [36, 37].

5 Conclusion

This study demonstrates that, in benzene-induced pre-leukemic rats melatonin provides dose-dependent protective benefits, with the most consistent improvement observed in CAT activity, while SOD and MDA showed relatively limited changes. The present findings provide in vivo evidence that benzene exposure downregulates Activin A and Follistatin transcripts in bone marrow tissue, and that melatonin (10 mg/kg) further suppresses these, compared with benzene, suggesting transcriptional modulation of this signaling axis. Protein biomarker responses varied among markers. Serum Activin A was not fully restored by melatonin, but Follistatin peaked at 20 mg/kg. Compared with previous melatonin treatments, 20 mg/kg yielded the lowest 8-OHdG levels and exhibited partial recovery of CAT, indicating that it provides a viable efficacy window. Together, melatonin partially restores redox status and reprograms Activin/Follistatin signaling without normalizing all endpoints and therefore identifies these transcripts and CAT activity as potential mechanistic targets for future investigation.

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