ORIGINAL ARTICLE

IN UTERO HYDROQUINONE EXPOSURE INDUCES LINEAGE-DEPENDENT OXIDATIVE STRESS IN MATERNAL HEMATOPOIETIC STEM/PROGENITOR CELLS

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ABSTRACT

In utero benzene exposure is associated with an increased risk of hematotoxicity; however, its effects on oxidative stress and lineage-specific alterations within the maternal hematopoietic stem/progenitor cell (HSPC) niche remain poorly defined. Hydroquinone (HQ), a reactive metabolite of benzene implicated in benzene-induced hematopoietic toxicity, was therefore examined in this study. Briefly, pregnant mice (n=18) were divided into 3 groups, comprising the control and HQ-treated groups. HQ was administered at 25 (HQ-25) and 50 (HQ-50) mg/kg body weight on gestational day (GD) 12, 14, and 16, followed by maternal bone marrow (BM) harvest on GD 18. Then, colony-forming unit (CFU) assays were performed to assess the clonogenicity status of myeloid, erythroid, and pre-B lymphoid progenitors. Colonies-derived CFUs were counted and harvested for oxidative stress profile. HQ caused a significant reduction in colony counts of pre-B lymphoid progenitors across all HQ-exposed groups and for erythroid progenitors at the 50 mg/kg dosage ($p<0.05$). However, the colony counts for myeloid progenitors are not significantly affected. Meanwhile, HQ significantly decreased ($p<0.05$) the glutathione (GSH) level only in myeloid progenitors, while no effect was observed on superoxide dismutase (SOD) level in other cell lineages. Oxidative stress markers were also influenced by cell lineages. HQ exposure significantly increased ($p<0.05$) lipid peroxidation in erythroid progenitor cells and showed no remarkable effect on protein oxidation for all cell lineages. Conclusively, HQ exposure can induce oxidative damage in the maternal HSPC niche, and the effect depends on hematopoietic cell lineages.

Keywords: *benzene, in utero toxicity, maternal, hematopoietic stem/progenitor cells, cell lineages, oxidative stress*

44 **Abbreviations:**
45 1,4-BQ: 1,4-benzoquinone
46 ANOVA: Analysis of variance
47 BFU-E: Burst-forming unit-erythroid
48 BM: Bone marrow
49 BSA: Bovine serum albumin
50 C_6H_6: Benzene
51 CFU-E: Colony-forming unit-erythroid
52 CFU-GM: Colony-forming unit-granulocyte macrophage
53 CFU-pre-B: Colony-forming unit-pre-B
54 CFU-S: Colony-forming unit-spleen
55 CLP: Common lymphoid progenitors
56 CMP: Common myeloid progenitors
57 CO_2: Carbon dioxide
58 CYP2E1: Cytochrome P2E1
59 DMEM: Dulbecco's Modified Eagle Medium
60 DNA: Deoxyribonucleic acid
61 DNPH: 2,4-Dinitrophenylhydrazine
62 DTNB: 5,5'-dithiobis-2-nitrobenzoic acid
63 NBT: Nitroblue-tetrazolium
64 EDTA: Ethylenediaminetetraacetic acid
65 FBS: Fetal bovine serum
66 GD: Gestation day
67 GSH: Derived compound glutathione
68 GSH: Glutathione
69 H_2O_2: Hydrogen peroxide
70 HBSS: Hank's Balanced Salt Solution
71 HCl: Hydrochloric acid
72 HQ: Hydroquinone
73 HSC: Hematopoietic stem cell
74 HSPC: Hematopoietic stem/progenitor cell
75 IARC: International Agency for Research on Cancer
76 ICR: Imprinting control region
77 IL: Interleukin
78 KTX: Ketamine, thiopentone, xylazine
79 LSC: Leukemic stem cell
80 MDA: Malondialdehyde
81 MPO: Myeloperoxidase
82 MPP: Multipotent progenitors
83 NaH_2PO_4: Sodium dihydrophosphate
84 NaOH: Sodium hydroxide
85 NK: Natural killer
86 OECD: Organization for Economic Co-operation and Development
87 PBS: Phosphate-buffered saline
88 PC: Protein carbonyl
89 Pen/strep: Penicillin/streptomycin
90 ROS: Reactive oxygen species
91 SCF: Stem cell factor
92 SEM: Standard error of mean
93 SOD: Superoxide dismutase
94 SPSS: Statistical Package for the Social Sciences
95 TBA: Thiobarbituric acid
96
97

98 **INTRODUCTION**

99 Benzene (C_6H_6) is a colourless liquid with an aromatic scent at room temperature. It is a widely used
100 solvent that serves as an essential starting material and intermediate in the production of chemicals and
101 gasoline formulations (U.S. Department of Health and Human Services, 2018). Then, human exposure to
102 benzene typically occurs among industrial workers who use solvents in the chemical industry, petroleum
103 refineries, oil pipelines, repair shops, and vehicle garages (McHale, Zhang & Smith, 2012). According to
104 previous studies, industrial workers exposed to benzene had significantly lower white and red blood cell
105 counts compared to the control group. This suggests that benzene toxicity to the hematopoietic system
106 may affect the function of blood cells of various lineages (Mathialagan et al., 2020). Benzene exposure
107 can cause significant health effects and is associated with an increased risk of blood diseases, including
108 leukemia, lymphoma, aplastic anemia, pancytopenia, and chromosomal aberrations (D'Andrea & Reddy,
109 2013). The International Agency for Research on Cancer (IARC) has classified benzene as a Group 1
110 carcinogen, which is carcinogenic to humans (IARC, 1987). Benzene enters the human body through
111 inhalation and the skin (D'Andrea & Reddy, 2013). Previous studies have reported that benzene
112 metabolite metabolism is the cause of benzene toxicity to bone marrow (Chow et al., 2018; Ross & Zhou,
113 2010). Benzene metabolism occurs in the liver, producing benzene oxide via cytochrome P450
114 (CYP2E1)-mediated oxidation. Benzene oxide will then undergo oxidation, forming reactive metabolites
115 such as phenol, hydroquinone (HQ), catechol, and benzoquinone (Mathialagan et al., 2020). The
116 hydrolysis of phenol to HQ by cytochrome P450 will cause HQ to accumulate in the bone marrow
117 (Enguita & Leitao, 2013).
118
119 Benzene metabolite, HQ, is a ubiquitous environmental contaminant due to its widespread use in human
120 and industrial activities. For example, HQ is used as a chemical in photography, a stabiliser in paint, and
121 a motor fuel. HQ is also used to produce antioxidants in food, rubber, plastics, and other industries
122 (Enguita & Leitao, 2013). HQ is a hematotoxic and carcinogenic agent associated with carcinogenesis
123 due to benzene exposure through occupational and environmental sector sources (OECD, 2009).
124 According to previous studies, HQ influences the formation of mononuclear leukemia cells, renal tubular
125 tumor cells, and liver cancer in study animals (Enguita & Leitao, 2013). Not only that, HQ can also
126 increase cell oxidative stress and affect cell viability (Bhattarai et al., 2020).
127
128 Bone marrow is the primary site of hematopoietic stem/progenitor cells (HSPCs), which maintain
129 hematopoiesis and ensure its stability and survival. This niche contains hematopoietic stem cells (HSCs) and
130 progenitor lineages that are capable of self-renewal and differentiation into mature, functional blood cells
131 (Mathialagan et al., 2020). HSCs give rise to multipotent progenitors (MPPs), which comprise common
132 myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs). These progenitors differentiate into
133 distinct lineages with specific functions. CMP generates granulocytes, monocytes, erythrocytes, and
134 megakaryocytes, supporting innate immunity and hemostasis, whereas CLP gives rise to T, B, and natural
135 killer (NK) cells for adaptive immunity (Brown, 2025). HSCs must proliferate and balance two opposing cell-
136 fate decisions, self-renewal and differentiation, to maintain the stem cell compartment and produce all the
137 needed downstream progenitors and mature blood cells. Under homeostatic conditions, HSCs cycle
138 infrequently and are primarily in a dormant state, quiescence (Wilson et al., 2008; Foudi et al., 2009). This
139 observation aligns with an earlier study demonstrating that lineage-biased MPPs acquire unique transcriptional
140 and metabolic profiles during commitment, which shape their proliferation capacity and responses to stress
141 (Vaníčková et al., 2023). Myeloid and erythroid progenitors often exhibit higher proliferative and oxidative
142 activity, whereas lymphoid progenitors display features that support DNA repair and redox control,
143 contributing to their differential susceptibility to toxic insults (Vaníčková et al., 2023). However, studies
144 concerning comparative toxicological assessment targeting differential lineages of hematopoietic progenitors
145 remain limited. Previous studies by Chow et al. (2021) and Dewi et al. (2023) performed comparative
146 toxicological assessments of benzene metabolite 1,4-benzoquinone (1,4-BQ) across different hematopoietic
147 progenitor lineages. Their findings demonstrated lineage-specific responses, where pre-B lymphoid
148 progenitors exhibited greater chromosomal aberrations, while alterations in microRNAs and transcription

factors regulating HSPC self-renewal and differentiation were observed across myeloid, erythroid, and lymphoid progenitors.

In 1961, Till and McCulloch developed the colony-forming unit-spleen (CFU-S) assay to detect and identify stem cells in mouse bone marrow (Siminovitch, McCulloch & Till, 1963). This assay is a hematopoietic functional assay often used to assess the function or potential of progenitors in stem cell products (Pamphilon et al., 2013). Benzene and its metabolites can induce oxidative stress and alter the HSPC microenvironment, leading to abnormal HSPC differentiation, increased apoptosis, DNA damage, and metabolic reprogramming. If damage to HSPCs persists, it can lead to the transformation into a leukemic stem cell (LSC) and, subsequently, to aplastic anemia and leukemia (Bhattarai et al., 2020). Not only that, reactive oxygen species (ROS) resulting from benzene exposure inhibit the cell cycle and induce oxidative stress, damaging HSPC macromolecules and ultimately disrupting hematopoiesis (Zhu et al., 2013). In the context of pregnancy, these effects may be exacerbated, as the maternal system undergoes substantial physiological adaptations from early gestation to delivery to accommodate both maternal and fetal demands (Akinlaja, 2016). Among the hematological changes that occur during pregnancy are increases in total blood volume and red blood cell mass, driven by elevated maternal erythropoietin production (Chandra et al., 2012). In addition, previous studies have reported increased bone marrow cellularity during pregnancy (El-Badri & Groer, 2008). Despite these physiological adaptations, exposure to benzene and its metabolites has been associated with the development of aplastic anemia and leukemia (Snyder, 2012). Although the occurrence of aplastic anemia and acute leukemia during pregnancy is rare, these conditions can lead to severe complications for both the mother and the fetus (Jaime-Pérez, González-Treviño & Gómez-Almaguer, 2021; Wang et al., 2021).

Previous studies have demonstrated the role of HSPCs of different lineages in mediating the toxic effects of benzene, particularly through targeting the HSPC niche in adult hematopoiesis (Chow et al., 2018; Chow et al., 2021; Dewi et al., 2023; Mohd Idris et al., 2018). However, studies investigating the toxic effects of HQ on maternal HSPCs remain limited. Given the distinct differences between normal adult and maternal hematological physiology, a deeper understanding of the mechanisms underlying benzene-induced maternal toxicity is essential. Therefore, this study was conducted to elucidate the toxic effects of HQ on bone marrow (BM) cells, with a particular focus on HSPC populations in pregnancy. The findings from this study may provide potential biological markers for risk assessment of toxic agents such as benzene and contribute to a more comprehensive understanding of how benzene exposure affects the maternal HSPC niche.

METHODS

Materials

The growth medium and growth factors for HSPCs used in this study were Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen Corporation, Carlsbad, CA, USA), supplemented with calf serum FBS (JRS Scientific Inc., Woodland, Canada), penicillin/streptomycin (100U/mL penicillin & 100 µg/mL streptomycin), stem cell factor (SCF), interleukin-3 (IL-3) and interleukin-6 (IL-6) (Miltenyi Biotec, Bergisch Gladbach, Germany). Other chemicals, such as phosphate-buffered saline tablets (PBS), Hank's Balanced Salt Solution (HBSS), hydroquinone (HQ), trypan blue powder, and bovine serum albumin (BSA), were purchased from Sigma-Aldrich (St. Louis, MO, USA). The methylcellulose media used for colony-forming unit (CFU) assays, namely MethoCult™ M3334, MethoCult™ M3534, and MethoCult™ M3630, were purchased from StemCell Technologies (Vancouver, British Columbia, Canada). Chemicals used in biochemical analysis include Triton X-100, Riboflavin, L-methionine, hydrochloric acid (HCl), sodium hydroxide (NaOH), sodium dihydrophosphate (NaH_2PO_4), ethylenediaminetetraacetic acid (EDTA), derived compound glutathione (GSH), 5,5'-dithiobis-2-nitrobenzoic acid (DTNB), hydrogen peroxide (H_2O_2), and Coomasie Brilliant Blue G-250 for Bradford staining.

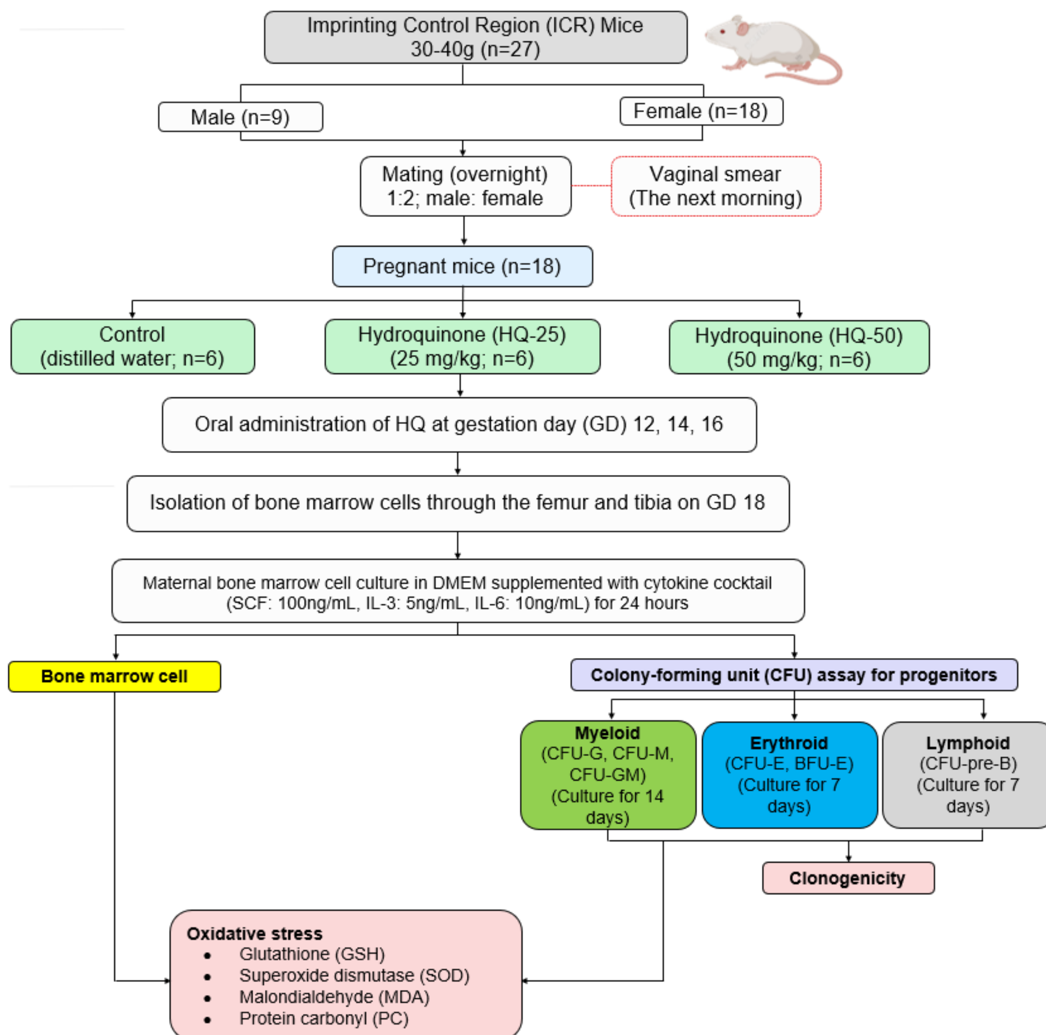
Experimental Design

The procedures involving the use of mice were approved by the Animal Ethics Committee of the

200 Universiti Kebangsaan Malaysia (UKMAEC, Kuala Lumpur, Malaysia) under approval number
201 FSK/2022/ZARIYANTEY/23-MAC/1237-MAC-2022-FEB-2025. A total of 27 Imprinting Control
202 Region (ICR) mice with male (n=9) and female (n=18) were used in this study. These ICR mice were
203 provided by the Animal Resource Unit, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia. Two
204 female mice were placed together with a male mouse for mating. Vaginal smear was done the next
205 morning to check for the presence of sperm. If sperm were detected, the day was counted as gestation
206 day (GD) 0 (Yusoff et al., 2021). Pregnant mice (n=18) were then placed in separate cages with one
207 mouse per cage. The pregnant mice were randomly divided into three groups (n=6/group), namely the
208 control group, the HQ-25 group, and the HQ-50 group. The control group received distilled water, the
209 HQ-25 group received HQ at 25 mg/kg body weight, and the HQ-50 group received HQ at 50 mg/kg
210 body weight (Mansell et al., 2019). HQ was given via oral gavage at GD 12, 14, and 16. These GDs were
211 selected to target mid-gestation, a period when maternal oxidative stress is known to increase as part of
212 physiological adaptation and when hematopoietic and immune systems undergo substantial changes,
213 making progenitor cells more susceptible to xenobiotic stress (Grzeszcza et al., 2023). On GD 18, the
214 pregnant mice were sacrificed by peritoneal injection of ketamine, thiopentone, and xylazine (KTX),
215 followed by isolation of BM cells from the femur and tibia using the flushing technique (Dewi et al.,
216 2023). The obtained BM cells were then pooled and filtered through a 40 μM nylon mesh (BD
217 Biosciences, San Jose, CA, USA) and suspended in DMEM media supplemented with 10% fetal bovine
218 serum (FBS), 2% penicillin/streptomycin (pen/strep), and cytokine cocktail (SCF: 100 ng/mL, IL-3: 5
219 ng/mL, and IL-6: 5 ng/mL). The BM cells were cultured in a standard cell culture incubator at 37°C in a
220 humidified atmosphere containing 5% CO_2 for 24 hours. Subsequently, the cells were subjected to
221 clonogenicity assays and oxidative stress profiling. The overall study design is presented in Figure 1.
222

223 **Clonogenicity Assessment**

224 A colony-forming unit (CFU) assay was performed according to the manufacturer's protocol
225 (StemCell Technologies, Vancouver, British Columbia, Canada). Three different semi-solid
226 methylcellulose media enriched with selective growth factors were used to culture the lineage-committed
227 hematopoietic progenitor cells. The analyzed progenitors were myeloid [colony-forming unit-granulocyte
228 macrophage (CFU-G, CFU-M, CFU-GM)] by using MethoCult media M3534. The MethoCult media
229 M3334 was used to culture erythroid progenitors [colony-forming unit-erythroid (CFU-E) and burst-
230 forming unit-erythroid (BFU-E)], and lastly, MethoCult media M3630 was used for pre-B lymphoid
231 clonogenicity [colony-forming unit-pre-B (CFU-pre-B)]. Briefly, freshly isolated single-cell suspensions
232 of BM cells were prepared, adjusted to 1×10^6 cells/mL, and incubated for 24 hours at 37°C with 5%
233 CO_2. After 24 hours of incubation, a cell count was performed to assess the cell viability. Subsequently,
234 cell suspensions with specific concentrations (2×10^5, 1×10^6, or 1×10^6 cells/mL) were prepared for
235 myeloid, pre-B lymphoid, and erythroid progenitors, respectively. These cell suspensions were mixed
236 with 1 mL methylcellulose media and then added to 6-well plates. These plates were then incubated at
237 37°C with 5% CO_2 for either 14 days for myeloid lineage or 7 days for pre-B lymphoid and erythroid
238 lineages. After incubation, the colonies formed in each CFU assay type were observed under an inverted
239 microscope, counted, and harvested for oxidative stress analysis.



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241
242 **Figure 1: Research design demonstrating the experimental workflow.**
243 Research design demonstrating the experimental workflow for the effects of HQ exposure via in vivo targeting
244 of BM cells and HSPCs of different lineages, comprising myeloid, erythroid, and pre-B lymphoid progenitors.
245

246 **Oxidative Stress**

247 In this study, four different oxidative stress profiles were analysed by using BM cells and
248 progenitor cells. For antioxidant status, glutathione (GSH) and superoxide dismutase (SOD) levels were
249 analysed, whereas for oxidative stress markers, malondialdehyde (MDA) and protein carbonyl (PC) were
250 analysed. Before performing oxidative stress profile analysis, cell lysates were prepared from BM cell
251 suspensions after 24 hours of incubation, and from HSPC suspensions after 14 days (myeloid) and 7 days
252 (lymphoid and erythroid) of incubation. Protein levels were also determined prior to the oxidative stress
253 analysis by using the Bradford assay. Measurement of GSH involves oxidizing GSH with 5,5-dithiobis
254 (2-nitrobenzoic acid) (DTNB), which can be detected at 415 nm. GSH levels were reported in terms of
255 mmol/mg (Ellman, 1959). For the assessment of SOD activity, the method relies on measuring the
256 inhibition of nitroblue-tetrazolium (NBT) reduction. The reduction of NBT by superoxide radicals
257 produces a blue formazan product, the intensity of which is measured at 560 nm. SOD activity is
258 expressed as U/min/mg (Beyer & Fridovich, 1987). Next, MDA quantification involves a reaction in
259 which one molecule of MDA reacts with two molecules of thiobarbituric acid (TBA) to form a
260 chromophore via extreme boiling (2,4-dinitrophenylhydrazine, DNPH). This results in precipitation of
261 the reaction protein and removal of excess DNPH at 100°C, followed by measurement at 532 nm. MDA

262 levels were reported in terms of nmol/g (Stocks & Dormandy, 1971). Lastly, the determination of PC
263 level was achieved by the reaction between protein oxidation and 2,4-dinitrophenylhydrazine. The
264 protein pellet was dissolved in a urea solution at 37°C, and the resulting supernatant was measured at 360
265 nm. PC levels were expressed in terms of nmol/g (Levine et al. 1990).
266

267 **Statistical Analysis**

268 Data were analyzed using statistical tests in the Statistical Package for the Social Sciences
269 (SPSS) version 26. The sample size for each group was n=6. All experimental data are expressed as mean
270 ± standard error of mean (SEM). Each analysis test was carried out in triplicate. The normality of the
271 data was first analysed using the Shapiro-Wilk test, followed by a One-Way ANOVA to compare the
272 mean values between the HQ-treated and control groups. A p-value of <0.05 indicates a significant
273 difference.

274

275 ## RESULTS

276 **Clonogenicity Assessment of Different Lineage-Committed Progenitors**

277 Colonies for lymphoid (CFU-pre-B) and erythroid (CFU-E, BFU-E) were observed after 7 days of
278 incubation, while colonies for myeloid (CFU-G, CFU-GM, CFU-M) were observed after 14 days of
279 incubation, as shown in Figure 2.
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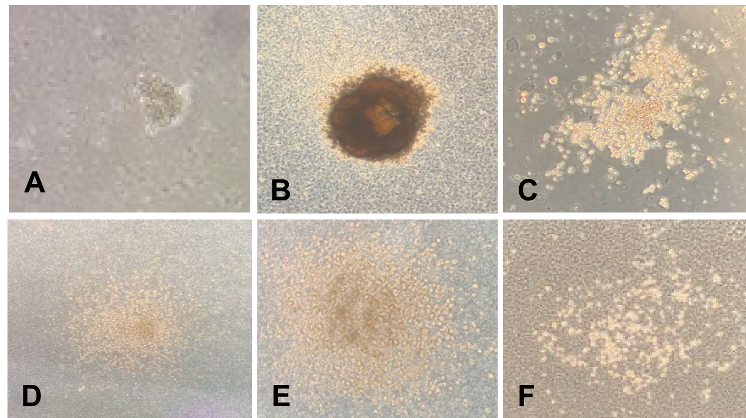

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289 **Figure 2: Microscopic images for hematopoietic stem/progenitor cells-derived colony-forming unit of**
290 **lymphoid, erythroid, and myeloid lineages.**
291 Colonies were observed under a microscope at 10x magnification for the morphology of (A) CFU-pre-B, (B) CFU-E,
292 (C) BFU-E, (D) CFU-G, (E) CFU-GM, and (F) CFU-M.
293

294 Figure 3 illustrates the clonogenicity profile of lineage-committed progenitors (pre-B lymphoid,
295 erythroid, and myeloid), as determined by colony counts after 7 and 14 days of incubation. For pre-B
296 lymphoid progenitors, a significant decrease ($p<0.05$) in colony numbers was observed following HQ
297 exposure at 25 mg/kg (14.00 ± 5.39) and 50 mg/kg (4.00 ± 1.92) compared with the control group
298 (43.60 ± 8.38). Similarly, erythroid progenitors showed a significant reduction ($p<0.05$) in clonogenicity
299 at 50 mg/kg (13.80 ± 3.02) relative to the control group (40.20 ± 8.70). However, the decrease at 25
300 mg/kg (16.80 ± 6.15) was not statistically significant ($p>0.05$). For myeloid progenitors, a reduction in
301 colony numbers was observed at both HQ concentrations compared with the control group.
302 Nevertheless, these decreases were not statistically significant ($p>0.05$) at 25 mg/kg (19.33 ± 3.84) and
303 50 mg/kg (21.17 ± 5.83) compared with the control (36.50 ± 6.17).
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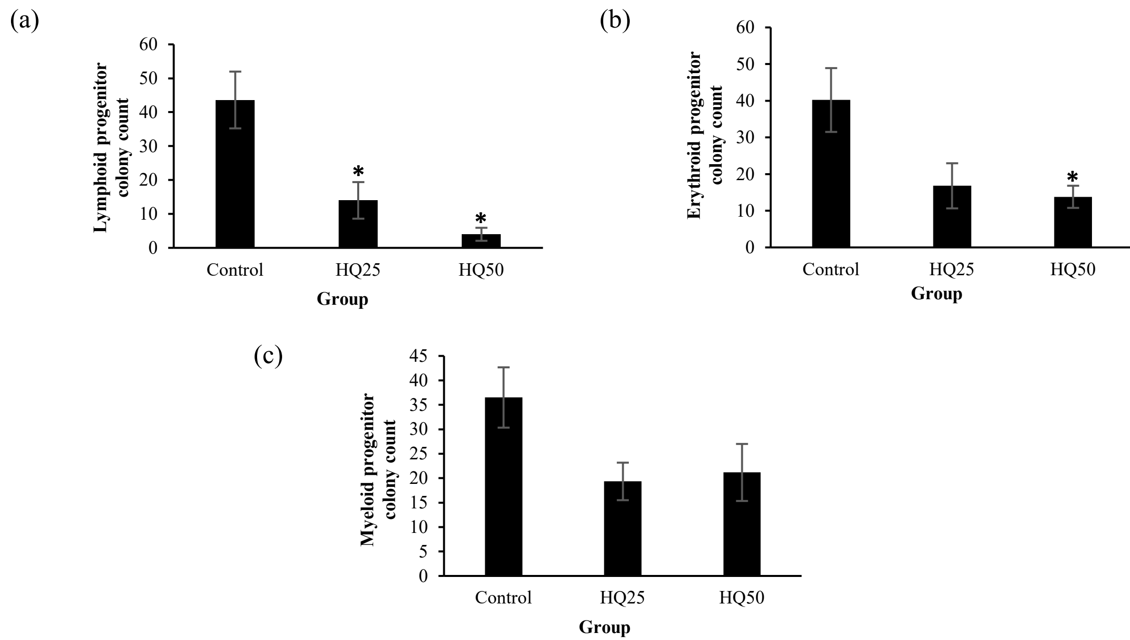

Figure 3: Effect of HQ exposure on the clonogenicity of differential lineage committed progenitors.
Effect of HQ exposure on the clonogenicity of (a) lymphoid progenitors, (b) erythroid progenitors, and (c) myeloid progenitors. Data are presented as mean ± SEM. *p<0.05 for significant differences between the control group and the HQ-treated groups.

Effect of HQ Exposure on Bone Marrow and Progenitors' Oxidative Stress Profiles

Figure 4 shows the effects of HQ exposure on bone marrow oxidative stress profiles. GSH antioxidant levels showed a non-significant decrease (p>0.05) between the control group (1.01 ± 0.31 nmol/g), the HQ-25 group (0.73 ± 0.21 nmol/g), and the HQ-50 group (0.93 ± 0.19 nmol/g). Similarly, SOD activity levels showed a non-significant (p>0.05) decrease for all HQ-treated groups, which are HQ-25 (7.34 ± 1.54 U/min/mg) and HQ-50 (5.29 ± 0.81 U/min/mg) compared to the control group (9.13 ± 1.34 U /min/mg) although there was a decrease for all HQ-treated groups. MDA levels also showed a non-significant difference (p>0.05) in MDA between the control group (86.62 ± 18.96 nmol/g) and the HQ-treated groups, namely the HQ-25 group (45.31 ± 8.90 nmol/g) and the HQ-50 group (148.28 ± 45.65 nmol/ g), despite showing remarkable differences between all study groups. Lastly, HQ exposure also did not show significant differences between the control group (2.89 ± 0.72 nmol/g), the HQ-25 group (3.51 ± 0.78 nmol/g), and the HQ-50 group (1.32 ± 0.28 nmol/g) for PC level.

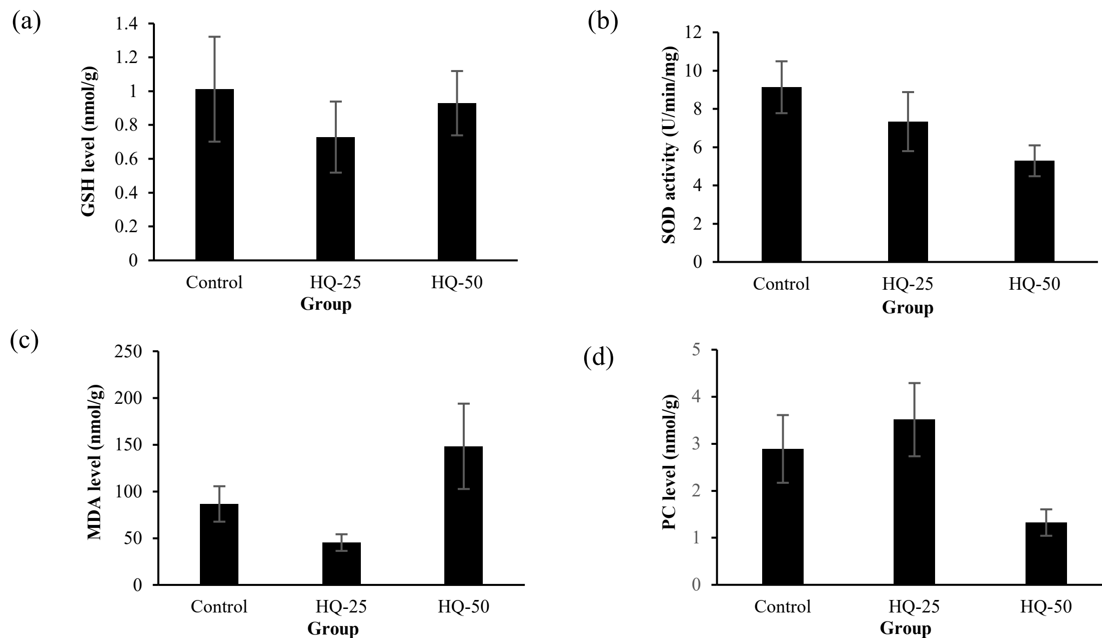

Figure 4: Effect of HQ exposure on BM cells' oxidative stress profiles.
Effect of HQ exposure on the BM cells, including (a) GSH level, (b) SOD activity, (c) MDA level, and (d) PC level. Data are presented as mean ± SEM.

Next, Figure 5 shows the effects of HQ exposure on the oxidative stress profiles of lymphoid progenitor cells. GSH level showed no significant differences ($p>0.05$) between the control group (0.43 ± 0.05 nmol/g), the HQ-25 group (0.26 ± 0.06 nmol/g), and the HQ-50 group (0.24 ± 0.07 nmol/g), although there was a decrease in the HQ-treated groups. The results of SOD activity also showed no significant difference ($p>0.05$) between the control group (12.03 ± 2.22 U/min/mg), the HQ-25 group (8.27 ± 2.59 U/min/mg), and the HQ-50 group (8.08 ± 2.02 U/ min/mg), although there was a decrease in the HQ-treated groups. Next, from the exposure of HQ on lymphoid pre-B progenitor cells, there was no significant difference ($p>0.05$) in the MDA level between the control group (54.21 ± 10.14 nmol/g), the HQ-25 group (154.67 ± 48.39 nmol/g), and the HQ-50 group (163.75 ± 42.93 nmol/g), although there was a significant increase in the HQ-treated groups. Lastly, PC levels on lymphoid progenitor cells also showed no significant differences ($p>0.05$) between the control group (1.39 ± 0.35 nmol/g) and all HQ-treated groups, namely the HQ-25 group (1.62 ± 0.43 nmol/g) and the HQ-50 group (0.78 ± 0.22 nmol/g), although there were remarkable differences between all HQ-treated groups.

Figure 6 shows the effects of HQ exposure on myeloid progenitor cells' oxidative stress profiles. Through the GSH level results obtained, it was found that there is a significant decrease ($p<0.05$) in GSH levels for all HQ-treated groups, namely the HQ-25 group (0.23 ± 0.06 nmol/g) and the HQ-50 group (0.25 ± 0.07 nmol/g) compared with the control group (0.68 ± 0.11 nmol/g). Next, from the results of SOD activity, there was no significant difference ($p>0.05$) between the control group (8.93 ± 0.92 U/min/mg), the HQ-25 group (8.96 ± 0.76 U/min/mg), and the HQ-50 group. (7.48 ± 1.56 U/min/mg). As for MDA levels on myeloid progenitor cells, there was also no significant difference ($p>0.05$) between the control group (16.51 ± 7.41 nmol/g), the HQ-25 group (28.64 ± 8.66 nmol/g), and the HQ-50 group (47.13 ± 15.75 nmol/g), although there was an increase among all HQ-treated groups. Lastly, for PC levels, there was no significant difference ($p>0.05$) between the control group (0.79 ± 0.17 nmol/g), the HQ-25 group (1.16 ± 0.42 nmol/g), and the HQ-50 group (0.61 ± 0.17 nmol/g).

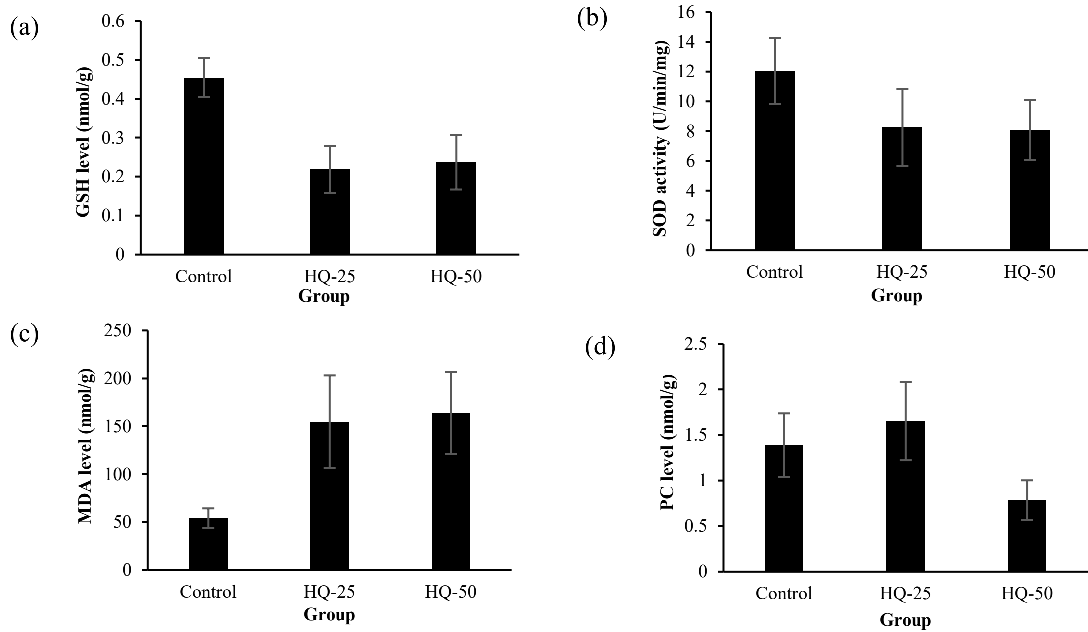

Figure 5: Effect of HQ exposure on lymphoid pre-B progenitor cells' oxidative stress profiles.
Effect of HQ exposure on the lymphoid pre-B progenitor cells, including (a) GSH levels, (b) SOD activity, (c) MDA level, and (d) PC level. Data are presented as mean ± SEM.

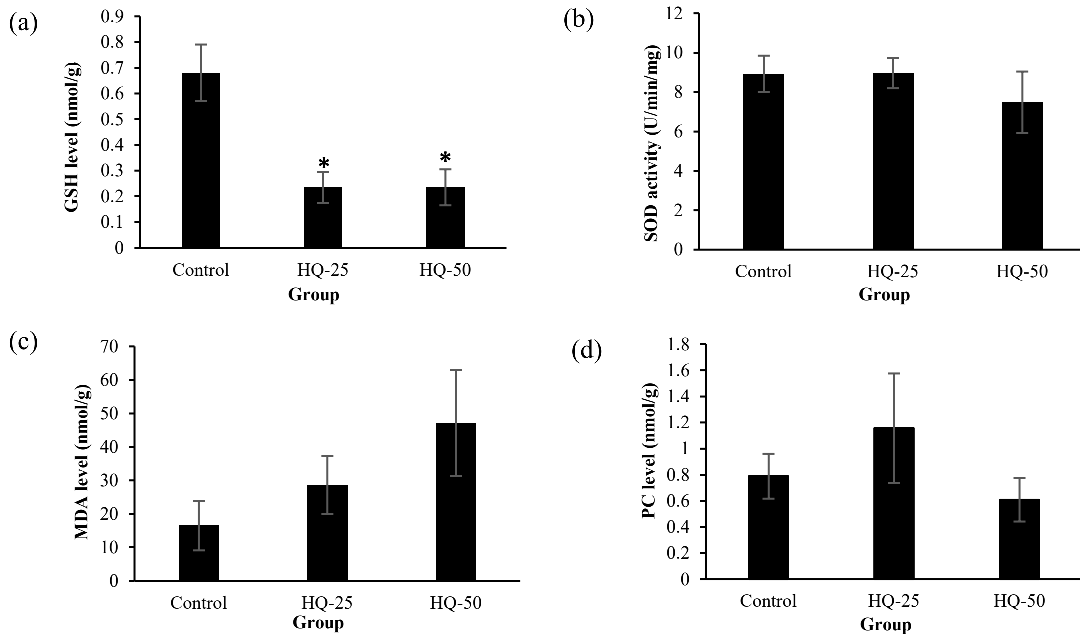

Figure 6: Effect of HQ exposure on myeloid progenitor cells oxidative stress profiles.
Effect of HQ exposure on the myeloid progenitor cells, including (a) GSH levels, (b) SOD activity, (c) MDA level, and (d) PC level. Data are presented as mean ± SEM. *$p<0.05$ for significant differences between the control group and the HQ-treated groups.

Lastly, Figure 7 shows the effects of HQ exposure on the oxidative stress profiles of erythroid progenitor cells. The GSH level in erythroid progenitor cells showed no significant difference (p>0.05) between the control group (0.07 ± 0.01 nmol/g), the HQ-25 group (0.06 ± 0.02 nmol/g), and the HQ-50 group (0.02 ± 0.005 nmol/g), despite the decrease in the HQ-treated groups. Next, for the SOD activity, there is also no significant difference (p>0.05) that can be seen between the control group (6.75 ± 1.67 U/min/mg) and all HQ-treated groups, which are the HQ-25 group (7.81 ± 1.96 U/min /mg) and the HQ-50 group (5.75 ± 1.29 U/min/mg). As for MDA level of erythroid progenitor cells, there was a significant increase (p<0.05) between the control group (16.45 ± 5.90 nmol/g), the HQ-25 group (86.63 ± 23.23 nmol/g), and the HQ-50 group (83.90 ± 19.92 nmol/ g). HQ exposure showed no significant difference (p>0.05) in PC levels between the control group (0.61 ± 0.14 nmol/g) and the HQ-treated groups, namely the HQ-25 group (1.01 ± 0.39 nmol/g) and the HQ-50 group (0.82 ± 0.09 nmol/g).

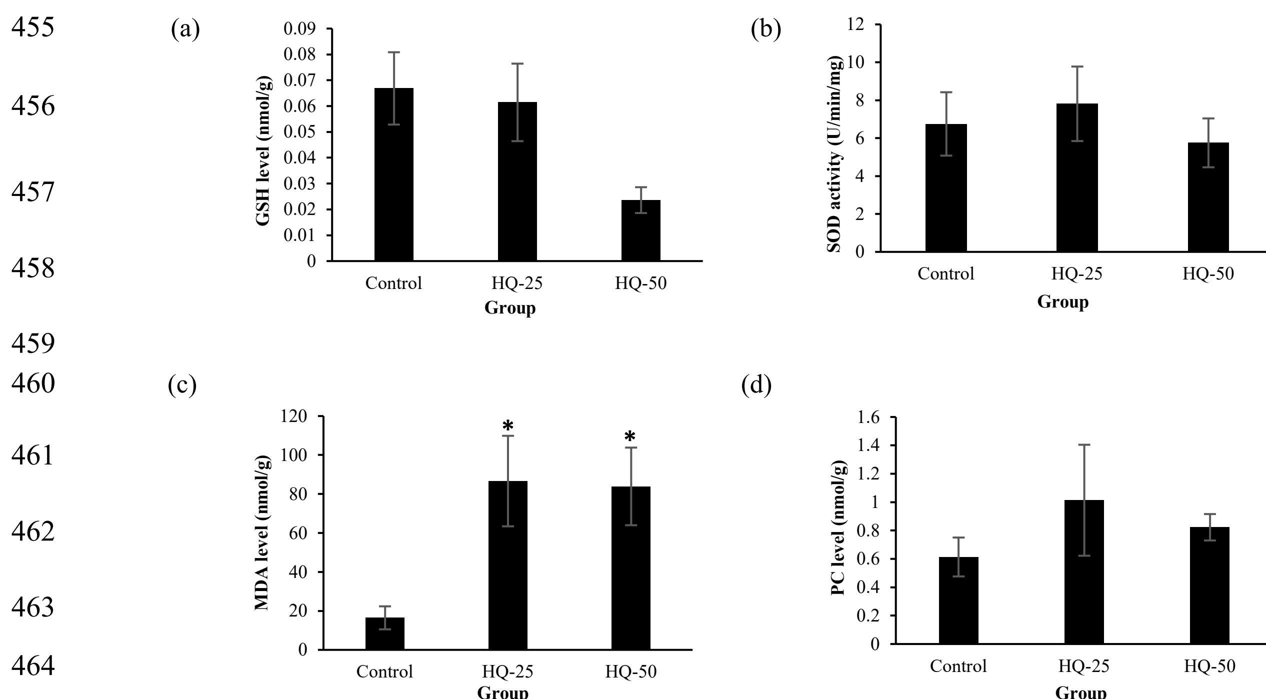

Figure 7: Effect of HQ exposure on erythroid progenitor cells' oxidative stress profiles.
Effect of HQ exposure on the erythroid progenitor cells, including (a) GSH levels, (b) SOD activity, (c) MDA level, and (d) PC level. Data are presented as mean ± SEM. *p<0.05 for significant differences between the control group and the HQ-treated groups.

DISCUSSION

IARC categorized benzene as a Group 1 carcinogen in 1987. Benzene and its metabolites can cause oncogenic lesions in the hematopoietic system and interfere with the normal processes of self-repair and HSPC differentiation (Chow et al., 2018). Among the benzene metabolites that are hematotoxic and carcinogenic agents is HQ (Enguita & Leitao, 2013). HQ can also be found in cigarettes and their smoke as well as diesel engines (Bando et al., 2017). According to Zhou et al. (2020), pregnancies exposed to benzene are very common, especially among pregnant women who smoke and mothers who are exposed to substances such as solvents, paints, and petroleum. Therefore, this study was conducted to examine the effects of HQ exposure on the maternal HSPC system using a clonogenicity assay and an oxidative stress profile.

In this study, two HQ concentrations (25 mg/kg and 50 mg/kg) were administered to mice in vivo. The HQ concentration of 50 mg/kg and the in vivo model were used in this study, following a previous study by Mansell et al. (2019), which examined DNA damage in umbilical cord blood and BM. Following this,

485 a lower dose of 25 mg/kg was included in the experimental design to assess dose-response relationships
486 and potential threshold effects on the studied parameters, in accordance with OECD Test Guideline 414,
487 which recommends the use of multiple dose levels to characterize toxicological responses across a range
488 of exposures (OECD, 2001). HQ administration was performed on gestational days (GD) 12, 14, and 16,
489 with the inclusion of GD 12 based on prior studies. Notably, Badham and Winn (2010) administered
490 benzene to maternal mice on GD 8, 10, 12, and 14 to examine HSC and fetal liver HSC development.

491 From the results of the colony count study for HSPCs of different lineages, the clonogenicity of lymphoid
492 progenitors showed a significant reduction for both concentrations of HQ (25 mg/kg and 50 mg/kg),
493 while erythroid progenitors showed a significant reduction only at the concentration of 50 mg/kg.
494 However, there was no significant decrease in myeloid progenitors. This contradicts a study by Mohd
495 Idris et al. (2018), which reported a significant reduction in the total number of myeloid progenitor
496 colonies. This may be due to differences in HQ administration between the previous and this study. Not
497 only that, although benzene targets all hematopoietic cell lineages, erythrocytes and lymphocytes are said
498 to be more affected by HQ (Corti & Snyder, 1998). This is evidenced by findings from a total colony
499 count study following HQ exposure, which showed a significant decrease in lymphoid and erythroid
500 progenitors. In the comparative analysis of HSPC lineages, erythroid progenitors showed the greatest
501 difference between the control and HQ induction groups. Benzene induces dramatic changes in erythroid
502 progenitor cells. For example, CFU-E cultured with various benzene metabolites is more sensitive than
503 other hematopoietic progenitor cells (Corti & Snyder, 1998). Because different types of blood cells have
504 different functions, the sensitivity of toxic substances and functional effects are also specific to cell types
505 (Mahalingaiah, Palenski & Van Vleet, 2018). These differences in lineage-specific sensitivity may be
506 attributed to distinct regulatory programs that govern transcriptional identity, differentiation kinetics, cell
507 cycle progression, and signaling dependencies across hematopoietic progenitor populations. Such
508 regulatory differences may underlie the observed variation in oxidative stress responses and clonogenic
509 outcomes among lymphoid, erythroid, and myeloid progenitors in the present study. Emerging evidence
510 from single-cell and genomic studies indicates that hematopoiesis follows a continuum of heterogeneous
511 progenitor states rather than a rigid hierarchical model, with erythroid, myeloid, and lymphoid
512 progenitors occupying distinct transcriptional and epigenetic trajectories (Laurenti & Göttgens, 2018).
513 These lineage-specific regulatory landscapes determine the activation of molecular pathways during
514 lineage commitment and, consequently, influence how progenitor cells respond to toxic perturbations
515 such as HQ exposure.

516 Next, HQ is a redox-active compound that can increase ROS formation, oxidative stress, and DNA
517 damage. Oxygen radicals produced during benzene metabolism can cause direct toxic effects, but, to a
518 lesser extent, they can also affect signalling pathways (Ross & Zhou, 2010). In general, oxidative stress
519 can be measured by measuring the products of oxidative damage reactions, such as lipid peroxidation and
520 protein oxidation. Not only that, but the assessment of antioxidant status can also be linked to oxidative
521 stress levels (Katerji et al., 2019). Therefore, this study used GSH and SOD assays to measure antioxidant
522 status, and MDA and protein carbonyl assays to measure oxidative stress markers in BM cells and HSPCs
523 after exposure to HQ. Based on the findings of the study, exposure to HQ did not cause significant
524 changes in GSH levels in BM cells, lymphoid progenitor cells, or erythroid progenitor cells, except
525 myeloid progenitor cells, which showed significant changes compared to the control group. Nevertheless,
526 there was a pattern of decrease in GSH levels in all cells. GSH is a non-enzymatic antioxidant involved in
527 various important cellular processes, including the antioxidant defence system, cofactor for enzymes,
528 xenobiotic detoxification, and protection of protein thiols from oxidation (Averill-Bates, 2023). This
529 pattern of decrease may be due to the nature of BM, which expresses myeloperoxidase (MPO) at high
530 levels, where MPO is reported to play a significant role in benzene-induced oxidative stress by oxidizing
531 HQ to reactive 1,4-BQ (Mathialagan et al., 2020).

532 This study also showed a non-significant decrease in SOD levels in BM cells and HSPCs. This
533 contradicts the study by Mathialagan et al. (2020), which showed a significant decrease in SOD levels in
534 the induction group compared to the control group after exposure to the benzene metabolite, 1,4-BQ, in
535 vitro. Nevertheless, there was a pattern of decline for each HQ induction group compared to the control
536 group. SOD is an antioxidant enzyme that regulates ROS by catalyzing the conversion of superoxide into
537 hydrogen peroxide and oxygen (Katerji et al., 2019). SOD is very important in protecting macromolecules
538 from the effects of benzene metabolites by catalyzing the dismutation of two superoxide anions into H_2O_2.
539 The results of decreased SOD levels in this study indicate that HQ suppresses antioxidants by producing

540 ROS, leading to oxidative stress.

541 Next, from the results of the MDA measurement, there was a significant increase in MDA levels in
542 erythroid progenitor cells between the control group and the HQ induction group. However, there were no
543 significant changes in BM cells or other progenitor cells, although there was a significant increase
544 between the control and HQ induction groups. According to Uzma, Kumar, and Zahari (2010), an
545 increase in ROS production will also increase lipid peroxidation, the main product of which is MDA. This
546 is in line with the findings of the MDA level of this study, which is that there is an increasing trend for
547 each HQ induction group compared to the control group, where the MDA level increases when the
548 concentration of HQ increases. Not only that, MDA and GSH levels are inversely related; that is, the
549 higher the MDA level, the lower the GSH level. High levels of MDA also indicate elevated free radical
550 levels and can suppress detoxification enzymes, such as GSH (Tualeka et al., 2019).

551 The results of the PC level measurement showed an increase in the HQ induction group at 25 mg/kg and a
552 decrease in the HQ induction group at 50 mg/kg. However, the changes that occur are not significant. This
553 finding is consistent with those of Mathialagan et al. (2020), who reported that oxidative stress products in
554 mouse BM cells decreased at high concentrations of 1,4-BQ compared to low concentrations. This may be
555 due to cytotoxicity and apoptosis in mouse cells induced by benzene at high concentrations. High
556 concentrations of benzene metabolites are highly reactive and can cause cell membrane damage and
557 apoptosis. Then, the increase in PC levels at HQ concentration can be caused by the oxidation of protein
558 side chains and amino acid residues by ROS, which further increases PC production (Katerji et al., 2019).
559 In addition, MDA resulting from lipid peroxidation can deactivate cellular proteins by forming protein
560 cross-links. The formation of these cross-links can promote protein aggregation, induce the accumulation of
561 oxidized proteins, and ultimately cause cellular dysfunction (Stadtman & Levine, 2000).
562
563 Non-significant findings in oxidative stress studies across multiple cell types can be due to several biological and
564 methodological factors. Different cells handle oxidative stress differently due to differences in baseline
565 antioxidant capacities within different types of cell lineage. Some cells naturally express higher levels of
566 antioxidant enzymes, and those cells with strong antioxidant defenses may neutralize ROS quickly, masking
567 measurable changes. In a similar context, in vivo oxidative stress is dynamic, and transient ROS elevations may
568 have been resolved through compensatory antioxidant responses prior to tissue collection. The protective role of
569 NAD(P)H: quinone oxidoreductase 1 (NQO1) against benzene-induced myelodysplasia in mice and
570 hematotoxicity in humans further supports the importance of detoxification pathways in limiting the persistence
571 and intensity of oxidative challenge in maternal HSPCs (Rothman et al., 1997). In addition, although the precise
572 contribution of individual benzene metabolites to benzene carcinogenicity remains unclear, the formation of 1,4-
573 BQ from HQ via MPO in the BM represents a key toxic pathway (Smith, 1996). Furthermore, cells with lower
574 mitochondrial activity produce less ROS, reducing detectable oxidative stress. In addition, differences in stressor
575 sensitivity cause some cells to activate protective pathways faster than others, restoring redox balance before
576 measurement. In terms of methodological factors, the concentration and exposure time of the treatment may not
577 generate enough oxidative stress. If the oxidative challenge is too mild, cells may not reach a measurable
578 oxidative imbalance. Then, high variability in cell isolation from different biological replicates may increase the
579 standard deviation and reduce statistical significance. Lastly, the limited sensitivity of conventional oxidative
580 stress markers may further contribute to non-significant outcomes (Halliwell & Whiteman, 2004). Therefore, real
581 biological effects may not reach statistical significance, even when functional changes are observed. Overall, as
582 described above, pooling or comparing differential cells together can dilute the statistical effect.
583
584 Exposure to HQ is known to increase intracellular ROS, leading to redox imbalance and oxidative damage to
585 cellular macromolecules, including lipids, proteins, and DNA. In the present study, the relatively modest changes
586 observed in oxidative stress markers (SOD, GSH, and oxidative damage markers) in whole BM cells likely reflect
587 the heterogeneous cellular composition of BM. The predominance of more resistant cell populations, particularly
588 myeloid cells, may dilute lineage-specific oxidative signatures, resulting in an overall attenuated oxidative stress
589 profile compared to that observed in progenitor subsets. At the progenitor level, however, differential sensitivity
590 to oxidative stress became more evident. Erythroid progenitors exhibited a marked increase in MDA levels,
591 indicating enhanced lipid peroxidation, accompanied by reduced GSH levels and decreased clonogenicity. This
592 suggests that HQ-induced ROS promotes membrane damage and disrupts redox homeostasis in erythroid-lineage
593 cells, which are known to be highly susceptible to oxidative stress due to their limited antioxidant capacity.
594 Similarly, lymphoid progenitors demonstrated significant functional impairment despite only moderate changes
595 in oxidative stress markers, indicating that even subtle redox disturbances may disrupt signaling pathways critical
596 for lymphoid proliferation and survival. In contrast, myeloid progenitors showed relative functional resistance,

597 with minimal changes in clonogenicity despite some evidence of antioxidant depletion, suggesting a greater
598 capacity to tolerate oxidative stress or more robust antioxidant defense mechanisms.
599
600 ROS function as critical modulators of HSPCs' behaviour within the BM microenvironment. Low ROS levels,
601 typically maintained in hypoxic niches, support stem cell quiescence and self-renewal by preserving genomic
602 integrity and promoting survival signaling pathways (Cieślar-Pobuda et al., 2017). Moderate ROS concentrations
603 act as physiological signals that facilitate controlled differentiation by activating lineage-specific transcription
604 factors. In contrast, excessive ROS accumulation induces oxidative stress, DNA damage, and the activation of
605 stress-response pathways, which can trigger apoptosis or skew differentiation. Alterations in ROS homeostasis
606 therefore disrupt the delicate balance between self-renewal, differentiation, and survival. Elevated ROS can
607 reduce stem cell pools, promote premature or aberrant differentiation, and increase cell death, whereas
608 insufficient ROS signaling may impair normal differentiation and immune cell development (Selvaraj et al.,
609 2025). By influencing these processes, ROS levels integrate intrinsic cellular programs with extrinsic
610 microenvironmental cues, highlighting their central role in maintaining hematopoietic homeostasis and functional
611 blood cell production.

612 Overall, in BM cells, HQ exposure caused modest but measurable changes in oxidative stress markers, including
613 a dose-dependent decrease in SOD activity, a modest reduction in GSH levels, and a marked increase in lipid
614 peroxidation at the higher exposure level. These effects were less pronounced and more variable than those
615 observed in lineage-committed progenitors, reflecting the heterogeneous nature of BM. Integration of oxidative
616 stress markers with lineage-specific clonogenic outcomes revealed clear differential sensitivity among HSPCs
617 following HQ exposure. Taken together, these findings demonstrate that HQ induces lineage-specific oxidative
618 stress responses that differentially translate into functional outcomes. While erythroid progenitors exhibit strong
619 biochemical evidence of oxidative damage, lymphoid progenitors appear to be the most functionally sensitive to
620 HQ exposure, likely due to their lower tolerance for redox imbalance. Myeloid progenitors show relative
621 resilience, consistent with known differences in hematopoietic lineage sensitivity to environmental toxicants.
622 This integrated analysis links redox disruption to progenitor-specific functional impairment, providing a
623 mechanistic framework for understanding HQ-induced toxicity in maternal HSPCs.
624

625 ## CONCLUSION

626 In conclusion, HQ exposure can induce oxidative damage in the maternal HSPC niche by generating ROS,
627 thereby disrupting the balance of self-renewal, differentiation, and survival within this microenvironment. The
628 severity of these effects varies by hematopoietic cell lineage, suggesting that HQ may cause selective
629 impairments in blood cell production that could adversely affect both maternal and fetal health.

630

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639

640 ## AUTHORS' CONTRIBUTION

641 Nur Afizah Y., Zariyantey A.H., and Farah Ezleen Aqilah A.B. formulated and designed the study. Farah
642 Ezleen Aqilah A.B. executed the experiments and prepared the data. Nur Afizah Y. and Farah Ezleen
643 Aqilah A.B. were responsible for methodology and validation. Nur Afizah Y. and Farah Ezleen Aqilah A.B.
644 were responsible for recruitment and sample collection. Nur Afizah Y., Farah Ezleen Aqilah A.B., and
645 Zariyantey A.H. played a key role in interpreting the results. Nur Afizah Y., and Farah Ezleen Aqilah A.B.,
646 drafted the writing of the manuscript. All authors actively contributed to providing critical feedback,
647 shaping the research, guiding the analysis, and refining the manuscript.

648

CONFLICT OF INTEREST DECLARATION

We affirm that there is no Conflict of Interest among the author(s) concerning the subject matter or materials discussed in this manuscript. We further certify that the article represents the original work of the Authors and Co-Authors. The manuscript has not been previously published and is not currently under consideration for publication elsewhere. This research/manuscript has neither been submitted for publication nor published in whole or in part elsewhere. We attest that all authors have made significant contributions to the work, ensuring the validity and legitimacy of the data and its interpretation, thereby warranting its submission to the Malaysian Journal of Clinical Biochemistry.

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