Research Article

Elucidating Lineage-Specific Myelotoxicity and Chromosomal Aberration Status in Hydroquinone-Exposed Hematopoietic Stem / Progenitor cells

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Abstract

Benzene exposure has been associated with hematotoxicity and leukemogenicity. However, the impact of benzene exposure on complex microenvironments of Hematopoietic Stem Cells (HSCs) niche, comprising of HSCs and lineage-specific progenitors remains elusive. Thus, a study on benzene-targeting HSCs niche could uncover mechanism linking benzene to HSCs niche alteration. This study evaluates the lineage-specific responses following exposure to a benzene metabolite, namely hydroquinone (HQ) in targeting HSCs and myeloid-committed progenitors. Freshly isolated murine bone marrow cells (BMCs) were exposed to HQ at series of concentrations (0–50 µM) for 24 hours; followed by cell viability analysis using MTT assay. Chromosomal aberration (CA) status was determined using karyotyping analysis. Expression of surface antigen for HSCs (Sca-1) was confirmed by flow cytometer. Lineage-specific myelotoxicity was studied using the colony-forming unit (CFU) assay for the following myeloid progenitors: CFU granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM), CFU-granulocyte/macrophage (CFU-GM), CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-erythroid (CFU-E) and Burst-forming unit erythroid (BFU-E). HQ reduced (p<0.05) viability of BMCs at 25 µM and 50 µM with the IC10, IC25, and IC50 were at 17 µM, 23 µM and 35 µM, respectively. Increased (p<0.05) frequency of CA was observed in HQ-treated group as compared to control. Reduced (p<0.05) Sca-1+ cells at 17 µM, 23 µM and 35 µM indicates cytotoxic effect of HQ targeting HSCs population. Reduced (p<0.05) total colony counts were noted following HQ exposure at 6.25 µM and a complete inhibition of colony growth were observed at higher concentration (12.5 µM). HQ reduced (p<0.05) the growth of CFU-GEMM, CFU-GM and CFU-G at 6.25 µM, while the growth of CFU-M, CFU-E and BFU-E were not remarkably affected at lower concentrations (1.56 µM and 6.25 µM). Conclusively, HQ induces chromosomal aberration and cytotoxicity on HSCs with notable lineage-specific responses in governing benzene-mediated myelotoxicity targeting HSCs niche.

Keywords: Benzene, Hematopoietic stem / Progenitor cells, Hematotoxicity, Lineage-dependent, Myelotoxicity

1.0 Introduction

Benzene is an important industrial material that is extensively used as a solvent and a known hematotoxicant (Khan., 2007; Zhang et al., 2012; IARC., 1987). Chronic occupational exposure to benzene has been linked to a number of hematological disorders and malignancies (Snyder et al., 1993). Despite of the reported findings, the precise mechanism mediating the events remains to be explored. Thus, it has been a major focus to study the mechanisms underlying benzene-induced hematotoxicity and leukemogenicity (Snyder, 2007; Atkinson, 2009; Bird et al., 2010; Wang et al., 2012). Hematopoietic stem cells (HSCs) have been shown as a potent target in benzene-induced hematotoxicity and leukemogenicity (Faiola et al., 2004). Exposure of HSCs to toxic and leukemogenic agents could lead to cytotoxicity and genotoxicity of the hematopoietic stem / progenitor cells, leading to hematopoietic disorders and malignancies.

In bone marrow (BM) compartment, the benzene metabolite namely hydroquinone (HQ) will be metabolized to 1,4-benzoquinone (1,4 BQ), which the metabolism is mediated by the activity of myeloperoxidase enzyme (MPO). The fundamental role of MPO in benzene metabolism causing the myeloid-committed progenitors becoming as a prime target for benzene toxicity as compared to other lineage-committed progenitors in Hematopoietic Stem Cells (HSCs) niche (Yoon et al., 2003). Thus, biotransformation of benzene to toxic metabolites plays the crucial role in mediating myelotoxicity, hematotoxicity and leukemogenicity affecting hematopoietic stem / progenitors niche (Faiola et al., 2004; Wang et al., 2012). However, understanding the mechanism linking benzene to the HSCs niche is challenging as the niche is a complex microenvironment composed of HSCs and committed progenitors for lymphoid, erythroid and myeloid lineages.

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Thus, novel approaches and mechanisms to address benzene toxicity at HSC niche targeting hematopoietic stem cells and progenitors of different lineages is fundamental.

Previously, our group has reported that 1,4-BQ mediates selective cytotoxicity targeting hematopoietic stem/progenitor cells via a lineage-dependent manner. The major highlights are 1) myeloid progenitors are more likely to be affected by the 1,4-BQ toxicity than the lymphoid progenitors and 2) 1,4-BQ demonstrates greater toxicity on restricted-progenitors than multi-potential progenitors (Chow et al., 2015). Moreover, 1,4-BQ exposure altered the expression of genes regulating self-renewal and differentiation pathway, indicating benzene potency to modulate the fate of hematopoietic stem/progenitor cells (Chow et al. 2018).

Despite of our reports, the impact of benzene exposure on HSCs niche deserve further investigations with particular interest on the role of other benzene metabolites in governing their toxic effects. Thus, the prime focus of this study is to elucidate potential effects of lineages-specific response in mediating benzene-induce myelotoxicity following exposure to a benzene metabolites, namely hydroquinone (HQ). With that, we employed a comparative investigation on the effect of HQ exposure targeting the myeloid-committed progenitors of different lineage commitment potency. This study utilized colony forming cells (CFC) assay for determination of multi-lineages-committed progenitors; regarded as colony forming unit (CFU) granulocyte/erythrocyte/macrophage/megakaryocyte (CFU-GEMM) and CFU-granulocyte/macrophage (CFU-GM) as well as single lineage-committed progenitors identified as CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-erythroid (CFU-E) and Burst-forming unit erythroid (BFU-E). Meanwhile, cytotoxicity and chromosomal aberration studies were carried out following HQ exposure to investigate the hematotoxicity and genotoxicity outcomes. Overall, the use of lineages-directed strategy can provide a fundamental platform in the study of benzene or other chemical toxicity targeting HSCs niche.

2.0 Materials and methods

2.1 Materials

The media and reagents to culture mouse bone marrow cells were as follows: Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen Corporation, Carlsbad, CA, USA), penicillin/streptomycin (PAA Laboratories GmbH, Pasching, Australia) and fetal bovine serum (FBS; JRS Scientific Inc., Woodland, Canada). Growth factors for hematopoietic stem/progenitor cells were comprised of stem cell factor (SCF), interleukin-3 (IL-3), and interleukin-6 (IL-6) (Miltenyi Biotec, Bergisch Gladbach, Germany). FITC-conjugated monoclonal Anti-mouse Sca-1+/Ly-6 and FITC-conjugated rat monoclonal IgG2a antibody were purchased from Miltenyi Biotec GmbH, Germany. HQ was obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Methylocellulose culture media were purchased from R&D System (HSC007). The reagents for the conventional karyotyping study were KaryoMAX Colcemid Solution, Tripsin EDTA 1x (Gibco, New York, USA) and Leishman stain (Sigma-Aldrich Co., St. Louis, MO, USA).

2.2 Isolation of mouse BM cells and hydroquinone (HQ) exposure

Mouse bone marrow cells (BMCs) were freshly isolated using flushing technique following protocol as described (Chow et al., 2015) and approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC Approval Number: FSK/2016/ZARIYANTEY/27-JULY/773-JULY-2016-JUNE-2017-AR-CAT2). Briefly, 10-week-old male imprinting control region-strained mice (ICR) were purchased from the Laboratory Animal Resource Unit, Faculty of Medicine, Universiti Kebangsaan Malaysia, Kuala Lumpur, Malaysia. Bone marrow cells were obtained through femur and tibia flushing technique. Collected BM cells were filtered through a 40 µm nylon mesh (BD Biosciences) and suspended in Hematopoietic Stem Cells growth media consisted of DMEM medium supplemented with 10% FBS, 1% penicillin/streptomycin, 100 ng/mL SCF, 10 ng/mL IL-6, and 5 ng/mL IL-3 (Chow et al., 2015; Chow et al., 2018). Cells were then maintained overnight in a humidified incubator at 37 °C and 5% CO2 prior to HQ exposure. Next, cells were treated with HQ at serial concentrations (1.5625, 3.125, 12.5, 25 and 50 µM) for 24 hours. The cells viability were determined using MTT assay following protocol as in Chow et al., 2015 and the IC50, IC10 and IC90 were obtained for downstream analysis.

2.3 Immunophenotyping of Sca-1+ cells

The effect of HQ exposure on HSCs population (Sca-1+) was confirmed using immunophenotyping analysis of surface marker expression for HSCs (Sca-1) through the application of BD FACSCalibur cytometer with Cell Quest Software (Becton, Dickinson and Company). Protocol for immunophenotyping was performed according to Chow et al., 2015. Briefly, following 24 hours of HQ exposure at IC10 (17 µM), IC25 (23 µM) and IC50 (35 µM), cells were harvested by centrifugation, suspended in 50 µl of staining buffer and loaded with fluorescein isothiocyanate (FITC) – conjugated anti-mouse Sca-1 antibody for 30 minutes incubation at 4°C in dark condition. Cells stained with the isotype matched antibody namely fluorescein isothiocyanate (FITC) – conjugated anti-mouse IgG2a were used as a control for the gating of positive cells. Both antibodies were from Miltenyi Biotec and applied at 0.1µg/1 x 10^7 cells. Mixtures were then washed with PBS, the supernatant was removed and the cells re-suspended into 400 µl PBS for the analysis using flow cytometer.

2.4 Colony forming cell (CFC) assay

Colony forming cell (CFC) assay was used to determine the effect of HQ exposure targeting various type of myeloid progenitors. CFC assay was done according to the protocol as described by Chow et al., 2015 using the Mouse Methylocellulose Complete Media (R&D System; HSC007), which supports the growth of the following myeloid progenitors: CFU-E, BFU-E, CFU-G, CFU-M, CFU-GM and CFU-GEMM. Briefly, after 24 h of HQ exposure (1.56, 6.25, 12.5 µM), the culture media from each of experimental group were discarded by centrifugation at 2500 rpm for 7 min. The cells were then re-suspended in fresh media, followed by cell viability count using trypan blue exclusion method. A total of 1 x 10^4 viable cells were then prepared in 100 µl of culture media and added to 1 mL of methylcellulose agar. The mixture was then seeded in a 35-mm plate. The cells were incubated at 37 °C with 5% CO2 for 14 days prior to colony scoring. During the culture period, the progenitor cell formed individual colonies that could be classified morphologically under inverted microscope. Meanwhile, the majority of terminally differentiated cells died out. Therefore, the presence of colonies indicated the presence of viable progenitor cells. The colonies were classified and enumerated based on the morphological recognition under inverted microscope on day 14 of culture. Image representing each CFUs is as shown (Figure 1).
Figure 1: Photographs representing the morphology of colonies for colony-forming units (CFUs) of respective progenitor. (A): CFU-E (erythroid); (B): BFU-E (Burst-forming unit erythroid); (C): CFU-G (granulocyte); (D): CFU-M (macrophage); (E): CFU-GM (granulocyte/macrophage); (F): CFU-GEMM (granulocyte /erythrocyte /macrophage /megakaryocyte) (Magnification: 40X)

2.5 Cytogenetic assay

Following exposure to HQ (1.5625, 3.125, 6.25 µM) and etoposide (0.05 µM; control positive) for 24 hours, 50µl colcemid (10µg/ml) was added to each culture 3 hours prior to harvesting in order to obtain a sufficient number of metaphase. Upon harvesting, the cells were centrifuged at 2500 rpm for 7 min which then followed by incubation with 0.075 M KCl hypotonic solution in 37°C water bath for 20 min. Then, cells were centrifuged at 2500 rpm for 5 min; followed by fixation with 2 ml fixative (methanol/glacial acetic acid, 3:1). Chromosomes observation was carried out using Leishman staining and the karyotyping was done by using GenASI's Bandview Software (Applied Spectral Imaging, Carlsbad, CA, USA). Briefly, twenty metaphases from the prepared slides were scored for every experimental group. The mouse chromosomes with total number of 40 including the chromosome X and Y were classified as normal chromosome and represented as 40,XY. Meanwhile, the chromosomal aberration (CA) status was determined according to the presence of structural (Robertsonian translocation) and numerical (aneuploidy) chromosomal abnormalities.

2.6 Statistical analysis

Each experiment was repeated three times, and the data are presented as the means ± standard error mean (SEM). Statistical analysis was conducted using SPSS version 16.0 (IBM Corporation, Armonk, New York, USA) and the level of significance used for all statistical tests was p < 0.05. One-way of variance (ANOVA) was conducted for comparison between two or more mean values between experimental groups.

3.0 Results

3.1 Cytotoxicity of HQ on bone marrow cells and hematopoietic stem cells (Sca-1+)

Significant reduction (p < 0.05) of cell viability was recorded for BMCs treated with HQ at 25 and 50 µM as compared to control (Figure 2a). The obtained IC$_{10}$, IC$_{25}$ and IC$_{50}$ for HQ were at 17, 23 and 35 µM; respectively. Meanwhile, exposure to HQ for 24 hours at 17, 23 and 35 µM causes significant reduction (p < 0.05) in the percentage of Sca-1$^+$ cells as compared to non-treated control group. Benzene is known hematotoxic and leukemogenic agents. However, the mechanism linking benzene exposure to the pathogenesis of hematological diseases and malignancies by targeting hematopoietic stem cells niche remain unclear. Previously, a number of studies have demonstrated the involvement of hematopoietic stem and progenitor cells (HSPC) as the prime target in benzene-induced toxicity (Zhang et al. 2012; Zhu et al. 2013; Chow et al. 2015; Sun et al. 2015). Therefore, studies investigating the impact of benzene metabolite exposure in targeting hematopoietic stem cells niche is fundamental in elucidating this underlying mechanism. Based on the viability analysis, HQ induced cytotoxicity on the bone marrow cells and HSC's population (Sca-1$^+$) which the effects are prominent following exposure to HQ at higher concentrations. Previously, exposure of mice to benzene through inhalation triggered cytotoxicity as demonstrated by reduction in the bone marrow cells and spleen cells cellularity (Green et al., 1981). Meanwhile, there was a notable increased in BMCs viability at low concentration of HQ (6.25 µM) exposure. Although the increment was not significant as compared to control, it may indicate differential response of cells. As reported by Hazel et al., 1996 exposure to low dose of HQ can inhibit apoptosis in the myeloblast and induce their proliferation, which can be associated to current finding.
Figure 2 (a) Cytotoxicity of HQ targeting bone marrow cells as determined by the MTT assay. Cells were exposed to HQ at a series of concentrations ranging from 0 to 50 µM for 24 h. (b) Cytotoxicity of HQ targeting hematopoietic stem cells population (Sca-1+) as confirmed using immunophenotyping analysis by flow cytometer. Cells were exposed to HQ at a series of concentrations at 0, IC10 (17 µM), IC25 (23 µM) and IC50 (35 µM) for 24 h. Data are presented as the mean ± S.E.M. (n=3). *p < 0.05 as compared to non-treated control group.

3.2 Effect of HQ exposure on clonogenicity of myeloid-committed progenitors

As shown in the Figure 3, exposure to HQ for 24 hours at respective concentrations inhibit clonogenicity of myeloid progenitors as evidenced by absent of CFUs formation at 12.5 µM (IC50), and significant (p < 0.05) reduction in CFUs counts at 6.25 µM exposure as compared to control.

Figure 3 The effect of HQ exposure on the number of colony-forming units (CFUs) of myeloid-committed progenitors. The mouse bone marrow cells were exposed to HQ for 24 h at 1.56 µM, 6.25 µM and 12.5 µM. The number of CFUs was counted under inverted microscope after 14 days of culture. Data are presented as the mean ± S.E.M. (n=3). *p < 0.05 as compared to non-treated control group.

3.3 Effect of HQ exposure on multi-lineages myeloid-committed progenitors

The effects of HQ exposure for 24 hours on the CFU-GEMM (a) and CFU-GM (b) are as shown (Figure 4). Overall, exposure to 12.5µM HQ causes complete inhibition of CFUs growth for both progenitors. Clonogenicity of CFU-GEMM was reduced following HQ exposure with significant reduction (p<0.05) of CFUs was noted at 1.56 µM and 6.25µM. Meanwhile, the clonogenicity of CFU-GM was significantly (p<0.05) reduced only at 6.25µM HQ as compared to control group.

Figure 4 Effect of HQ on the clonogenicity of multi-lineages myeloid-committed progenitors was determined according to the colony counts of (a) CFU-granulocyte, erythroid, macrophage and megakaryocyte (CFU-GEMM) and (b) CFU-granulocyte and macrophage (CFU-GM). The mouse bone marrow cells were exposed to HQ for 24 h at 1.56 µM, 6.25 µM and 12.5 µM. The number of CFUs was counted and classified morphologically under inverted microscope after 14 days of culture. Data are presented as the mean ± S.E.M. (n=3). *p < 0.05 as compared to non-treated control group.
3.4 Effect of HQ on single-lineages myeloid-committed progenitors

The responses of single-lineages myeloid-committed progenitors toward HQ exposure for 24 hours are as shown in Figure 5. Overall, exposure to HQ at 12.5 µM inhibits CFU growth of every progenitor. The clonogenicity of CFU-G (a) and CFU-M (b) were reduced in a dose-dependent manner, with a significant (p<0.05) reduction was only notable for CFU-G at 6.25 µM as compared to control group. Meanwhile, the BFU-E (c) showed differential response with a notable slight increase in CFUs count at 1.56 µM HQ exposure, which was further reduced following an exposure to higher concentration (6.25 µM). As for CFU-E, no remarkable changes in CFUs growth was identified for HQ-treated group at 1.56 µM and 6.25 µM than the control.

![Graphs showing clonogenicity and BFU-E counts](image)

Figure. 5 Effect of HQ on the clonogenicity of single-lineages myeloid-committed progenitors was determined according to the colony counts of (a) CFU-granulocyte (CFU-G), (b) CFU-macrophage (CFU-M), (c) Burst-forming unit erythroid (BFU-E) and (d) CFU-erythroid. The mouse bone marrow cells were exposed to HQ for 24 h at 1.56 µM, 6.25 µM and 12.5 µM. The number of CFUs was counted and classified morphologically under inverted microscope after 14 days of culture. Data are presented as the mean ± S.E.M. (n=3). *p < 0.05 as compared to non-treated control group.

HSCs and hematopoietic progenitors possess the ability to proliferate and differentiate into mature cells. This unique ability enables them to form colonies in a semi-solid medium. Thus, the colony-forming unit (CFU) assay has been widely used for the toxicological assessment targeting HSCs and progenitors (Parent-Massin, 2001; Malerba et al., 2004). A previous in vivo study reported that benzene reduces the number of multi-potential hematopoietic stem cells (CFU-S) and of colony-forming units-granulocytes and macrophages (CFU-GM) in CD-1 mice (Green et al., 1981). Thus, they showed that depletion of stem cells is involved in the pathogenesis of benzene-induced hematotoxicity. Furthermore, Corti and Snyder (1998) reported that some of the benzene metabolites, such as HQ, phenol, and 1-BQ, exhibited dose-dependent inhibition in colony growth of mouse colony-forming units-erythroid (CFU-E). The effects of benzene on the clonogenicity of HSCs and HPCs can generate information on their capacity for proliferation and multipotency, which are crucial for the maintenance of hematopoiesis. Current finding demonstrated that myeloid progenitors are responsive to HQ exposure in lineage-specific manner. CFU-GEMM which represent the most highly primitive myeloid progenitors with multi-lineages commitment potency showed greater susceptibility to HQ toxicity as evidenced from significant reduction in CFU-GEMM counts following exposure to HQ at as low as 1.56 µM. In contrast, the clonogenicity for the majority of single-lineages myeloid-committed progenitors (CFU-M, CFU-E and BFU-E) were significantly impaired only at higher concentration of HQ exposure (12.5 µM). This study emphasizes the lineage-specific responses of various myeloid-committed progenitors which the finding could uncover potential mechanism linking benzene to myelotoxicity targeting HSCs niche.

3.5 Effect of HQ exposure on the chromosomal aberration status of marine bone marrow-derived HSCs

Chromosomal aberration status was identified following 24 hours exposure to HQ and Etoposide. Images representing structural and numerical abnormality of the chromosome for every experimental group are as shown in Figure 6. A total of 31.7 ± 6.0%, 23.3 ± 4.4% and 38.3 ± 8.8% of chromosomal abnormalities were detected (Figure 7) at 1.56µM, 3.12µM and 6.25µM HQ; respectively as compared to positive control group treated with etoposide (41.7 ± 10.1%). The percentage of chromosomal abnormality was significantly (p <0.05) higher following HQ exposure as compared to non-treated control group which presented with non-detectable chromosomal abnormality.
Figure 6: The spread of chromosome metaphase and arrangement of chromosomes (1-19 pairs and XY) in the karyotyping software. Images representing structural and numerical abnormalities of the chromosome following HQ and etoposide exposure for 24 h. Each metaphase is analyzed using Applied Spectral Imaging System. The chromosome analyzed based on Adler ideogram. A: Control - Karyotype:40,XY, B: 1.5625µM HQ – Karyotype:39,XY,Rb(1.11), C: 3.125µM HQ - Karyotype:39,XY, Rb(1.11), D: 6.25µM HQ - Karyotype:39,XY, Rb(1.11), E: 0.05µM Etoposide - Karyotype:39,XY,Rb(1.11) F: 0.05µM Etoposide - Karyotype:37,XY,Rb(1.13)Rb(3.18),-19,-20, G: 1.5625µM HQ - hyperdiploidy karyotype, H: 6.25µM HQ - hyperdiploidy metaphase spread and I: Etoposide - hyperdiploidy metaphase spread (Magnification: 100X). Rb: Robertsonian.

Figure 7 Effect of HQ exposure on the chromosomal aberration status of murine bone marrow-derived HSCs following 24 h exposure to HQ and Etoposide. Data are presented as the mean ± S.E.M (n = 3). * p < 0.05 compared with the control group.
The mechanism of benzene-induced hematological diseases and malignancies remain unclear. A number of epidemiological studies have reported the presence of chromosomal aberration (CA) such as translocation and aneuploidy in peripheral lymphocytes isolated from exposed workers (Smith et al. 1998; Zhang et al. 1999; Zhang et al. 2011; Zhang et al. 2012). According to Smith (2010), a number of reactive benzene metabolites such as 1,4-benzoquinone (1,4-BQ) and hydroquinone (HQ) may cause CA, mitotic recombination aberration and epigenetic alteration in hematopoietic stem / progenitor cells. The event may lead to further genetic mutation affecting genes crucial for self-renewing and differentiation properties of hematopoietic stem / progenitor cells. In this study, the effects of HQ on chromosomal aberration status of exposed bone marrow cells was determined through karyotyping. Our study found that exposure to HQ at all tested concentrations able to induce significant chromosomal abnormalities in the exposed cells. Our result is in line to Ji et al (2009) who has shown that HQ treated human lymphoblastic cells (TK6) acquired structural and numerical abnormalities of the chromosome, indicating the genotoxic effect of HQ. Meanwhile, previous study has reported that lymphocyte of benzene-exposed workers showed the presence of micronuclei with HQ showed the ability to promote clastogenic and aneugenic effects in humans as compared to other benzene metabolites such as catechol, phenol and 1-4 benzoquinone (1.4 BQ). While there are reports concerning HQ genotoxicity, studies focusing on the effect of HQ exposure targeting hematopoietic stem cells niche particularly following exposure to non-cytotoxic concentrations remains to be explored. Our study demonstrates that HQ able to induced CA in mouse bone marrow cells even following exposure at non-cytotoxic concentrations (1.5625, 3.125, 6.25 µM) for 24 hours. In conclusion, future study focusing on genotoxic effect of benzene metabolite targeting progenitors of various lineages can provide fundamental approach to study benzene-mediated hematotoxicity and leukemogenicity targeting hematopoietic stem cells niche.

4.0 Conclusion

Conclusively, HQ able to induce chromosomal aberration and cytotoxicity on myeloid- committed progenitors with notable lineage-specific responses in governing benzene-mediated myelotoxicity targeting HSCs niche.

5.0 Declaration

The authors declare no conflicts of interest in this work.

6.0 Acknowledgements

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