Ethanolic Extract of Black Rice ‘Sembada Hitam’ Bran Protects the Cytotoxic Effect of H\textsubscript{2}O\textsubscript{2} on NIH3T3 Cells.

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Abstract
Oxidative stress which is triggered by endogenous and exogenous stressors such as oxygen metabolism in mitochondria, radiation, drugs, and pollutants, negatively affect biological systems. Various pathophysiological conditions and the life span of organisms were affected by such condition. Secondary metabolites found in natural ingredients such as black rice have high antioxidant activity that can prevent oxidative stress. This study aimed to examine potency of the ethanolic extract of black rice (\textit{Oryza sativa} L. ‘Sembada Hitam’) bran to protects H\textsubscript{2}O\textsubscript{2}-induced NIH3T3 cells. This research was focused to evaluate the potency of black rice bran’s (BRB’s) extract on cytotoxicity, apoptosis, and cell growth due to H\textsubscript{2}O\textsubscript{2} induction. This study used a combination of H\textsubscript{2}O\textsubscript{2} exposure at various concentrations (50, 100, 150, 200, and 300 μM) and BRB’s extract at various concentrations (7.81; 15.63; 31.25; 62.5; 125; 250; 500; and 1000 μg/mL). Our results showed that BRB’s extract at the concentration of 7.81 to 1000 μg/mL maintained NIH3T3 cells viability above 80% against 50 and 100 μM H\textsubscript{2}O\textsubscript{2} exposure for 24 hours. These were in line with the apoptosis test results, which showed that the BRB’s extract suppressed apoptosis, especially the combination of BRB and H\textsubscript{2}O\textsubscript{2} exposure at 62.5 μg/mL and 100 μM; 62.5 μg/mL and 200 μM, as well as 250 μg/mL and 100 μM, respectively. Moreover, the H\textsubscript{2}O\textsubscript{2}-induced NIH3T3 cells' growth was maintained up to the fifth day under the BRB’s extract treatment. The result proved that pretreatment of BRB’s extract at the concentration of 62.5 μg/mL is highly effective as an anti-apoptotic and increases cell proliferation up to the fifth day on H\textsubscript{2}O\textsubscript{2}-induced NIH3T3 cells. Collecting all the results together, we suggested that BRB’s extract have a protective effect by maintaining NIH3T3 cell viability against H\textsubscript{2}O\textsubscript{2} induction.
Introduction
Exposure of endogenous stressors through mitochondrial metabolic processes and exogenous stressors such as nutrients, lifestyle, chemical reagents, UV light, air pollution, molecular radiation, and microbiome can cause oxidative stress. An imbalance of oxidants and antioxidants that triggers excessive ROS (reactive oxygen species) production induces oxidative stress which leads to signaling disturbances and various biomolecules damaged such as the disturbance of genome integrity to redox metabolism that affects biological processes. Hydrogen peroxide (H$_2$O$_2$) are ROS that are produced mainly as byproducts of oxygen metabolism by mitochondria that act as cell signaling molecules to trigger adaptive responses that contribute to life extension. ROS production increases significantly above the antioxidant defense capacity, causing antioxidant activity in biological systems decrease. It will result in oxidative damage leads to cell dysfunction and behavioral changes that consequences in premature senescence, abnormal proliferation, deregulation of inflammatory responses, cell tumorigenesis, and carcinogenic processes. Excess of H$_2$O$_2$ exposure causes oxidative stress and decreases cell proliferation as well as cell viability leading to cell death.

Various studies showed that secondary metabolites in plants such as black rice from many cultivars (Oryza sativa L. ‘Cempo Ireng’), Oryza sativa L. Indica, Melik black rice, and Toraja local black rice have high antioxidant activity, which can eliminate oxidative stress. A high levels of nutrients in black rice such as protein, gluten, low sugar, fat, vitamin B, riboflavin, thiamine, vitamin E, iron, tocopherol, magnesium, niacin, phosphorus, zinc, and dietary fiber. Consumption of black rice can increase human life span, reduce inflammation and irritation, prevent anemia, treat various diseases (blood pressure, colds, urinary tract infections, heart attacks), and potentially as antiaging, anticancer, antidiabetic, as well as reduce the risk of obesity.

‘Sembada Hitam’ is one of Indonesian cultivars of black rice planted in Yogyakarta. Previous study showed 2.5 μg/mL of the ethanolic extract of BRB (black rice bran) ‘Sembada Hitam’ contained high antioxidants, which significantly reduces MDA (malondialdehyde) levels in HUVEC (human umbilical vein endothelial cells) induced by preeclampsia for 24 hours. The preeclampsia-induced MDA level of 8.7085 μM fell to 5.0895 μM, comparable with MDA control levels in normal pregnancy. On the other hand, the ethanolic extract of ‘Sembada Hitam’ rice bran was reported to have a potential antiangiogenic effect in preeclampsia treatment. However, the potency of ‘Sembada Hitam’ rice, especially in protecting cells from oxidative stress, has not been widely studied yet.

Materials and Methods
Extraction of Black Rice Bran
Black rice ‘Sembada Hitam’ purchased from the local farmer in Ngaglik, Sleman, Yogyakarta, Indonesia. Ten milligram of the bran was extracted by maceration with 85 mL of absolute ethanol (Sigma-Aldrich) and 15 mL of 1N HCl for 48 hours. The filtrate underwent remaceration twice overnight. Further, the filtrate was evaporated as described previously with slight modification.

Cell Culture
NIH3T3 cells line obtained from the Laboratory of Parasitology, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia. This study was conducted under Ethical Number: KE-FK-1351-EC-2021. The cells were cultured using DMEM and purchased from Gibco (made in USA) with REF number 12800-058. DMEM for culture containing 10% fetal bovine serum (FBS) (Origin: Brazil, REF number 10270-106), 2% penicillin streptomycin (REF number 15140-122), and 0.5% amphotericin B (fungizone) (made in Israel, REF number 15290-018). Cells were harvested after 80% confluence using PBS for washing and 0.5% trypsin-EDTA (made in Canada, REF number 25200-056).

MTT Assay
NIH3T3 cells were cultured in 96-well plate with a density 3500 cells/well using DMEM and incubated for 24 hours at 37 °C with 5% CO$_2$. After incubation, the cell was treated with various concentration (50; 100; 200; 300; 400; or 600 μM) of H$_2$O$_2$ for 24 hours with three replications. The cell also treated with the various concentration (7.81; 15.63; 31.25; 62.5; 125; 250; 500; or 1000 µg/mL) of ethanolic extract of black rice ‘Sembada Hitam’ bran for 24 hours with three replications. Cytotoxicity study was performed with the combination of selected H$_2$O$_2$ concentration which caused reduction on the...
viability of NIH3T3 cells (50, 100, 150, 200, or 300 μM), and ethanolic extract of black rice ‘Sembada Hitam’ bran at the various concentration (7.81; 15.63; 31.25; 62.5; 125; 250; 500 or 1000 μg/mL), then each three replication incubated for the next 24 hours. Cytotoxicity and cytoprotective studies was performed by MTT assay. The percentage of cell viability was calculated based on the Cancer Chemoprevention Research Center formula.\(^\text{18}\)

**Apoptosis Assay**
NIH3T3 cells were cultured in 24-well plate with a density 75000 cells/well and incubated for 24 hours at 37°C with 5% CO\(_2\). Cells were treated with combination of selected H\(_2\)O\(_2\) concentration (100 or 200 μM) and black rice bran’s extract selected concentration (62.5 or 250 μg/mL) for 24 hours with three replications. The cells were stained with 10 μL of acridin orange and propidium iodide AO/PI (1:1) on a cover slide for apoptotic detection. The green nuclei detected as a living cell and red nuclei as an apoptotic cell. A population of at least 200 cells per slide under a fluorescence microscope (Carl Zeiss/Axio Observe 21) were counted. The number of apoptotic cells were represented as percentage.

**Cell Growth Assessment by Trypan Blue Staining**
NIH3T3 cells were cultured in 6-well plate with a density 40000 cells/well and incubated for 24 hours at 37 °C with 5% CO\(_2\). After 24 hours incubation, the combination of selected H\(_2\)O\(_2\) concentration (100 or 200 μM) and black rice bran’s extract selected concentration (62.5 or 250 μg/mL) were treated on NIH3T3 cells with two replications. The H\(_2\)O\(_2\) induction was done for 24 hours whereas the black rice bran’s extract was treated continuously for another 5 days with a single passage after 3-days culture. The cells were counted at the 3rd and 5th day after trypsinization using trypan blue staining to analyze cell growth assay.

**Statistical Analysis**
Statistical data analysis was done by one-way analysis of variance (ANOVA) (p \(\leq\) 0.05) followed by Tukey HSD test. The results were described as mean ± standard deviation (SD).

**Results and Discussion**

**Cytotoxicity of Hydrogen Peroxide on NIH3T3 cells**
Increasing levels of ROS from various endogenous and exogenous stressors such as hydrogen peroxide (H\(_2\)O\(_2\)) induces lipid peroxidation or DNA oxidation which modifies cellular biomolecules and causes oxidative stress.\(^\text{19}\) We investigated the effect of H\(_2\)O\(_2\) exposure at various concentrations on NIH3T3 cell viability. The results showed that induction of 25 to 600 μM H\(_2\)O\(_2\) for 24 hours decreased the viability of NIH3T3 cells dose-dependent manner, especially

![Graph](image1.png)

*Fig. 1: H\(_2\)O\(_2\) exposure for 24 hours decreased NIH3T3 cell viability dose dependent manner. Each bar represents different treatment. The value in each bar represent the mean ± SD (standard deviation), triplicates. The different letters in each bar represents significant difference of the value (p \(\leq\) 0.05).*
100 to 600 μM H$_2$O$_2$ showed significantly different results from the control. Three hundred μM H$_2$O$_2$ exposure significantly decreased cell viability up to 40%, whereas at 600 μM, increased cell death (Figure 1.). Our data indicated that H$_2$O$_2$ was toxic by decreasing the viability of NIH3T3 cells.

The previous study also reported that H$_2$O$_2$ exposure to fibroblasts reduces the viability of MEF (mouse embryonic fibroblast) cells by around 30% at the concentration of 400 and 600 μM after 24 hours exposure, around 60% in human dermal fibroblasts (BJ cells) (500 μM of H$_2$O$_2$ exposure), around 60% in H8F2p25LM cells (10-100 μM H$_2$O$_2$ exposure), and around 60% in MRC-5 cells (100-500 μM H$_2$O$_2$ exposure). 70, 80, 90, and 100 μM H$_2$O$_2$ exposure cause the lethal condition on H8F2p25LM cells20. NOX (NADPH oxidase) enzymes are activated at the ligand-receptor interactions (TNFα-TNFR or EGF-EGFR), and extracellular superoxide dismutase (SOD3) captures superoxide, providing H$_2$O$_2$ for imported by aquaporins (peroxiporins). This condition triggers proliferation, migration, and angiogenesis.1 Cytotoxicity of Black Rice ‘Sembada Hitam’ Bran’s Extract on NIH3T3 cells

In this study, exposures of H$_2$O$_2$ induced oxidative stress and decreased cell viability to cell death. Natural ingredients, such as black rice, contains high antioxidant activity and protect cells from oxidative stress. The results showed that 7.81 up to 1000 μg/mL of black rice bran extract’s had no cytotoxicity effect on NIH3T3 cells, especially at the concentration of 250 to 1000 μg/mL which showed highly cell viability. These results provided 7.81 to 1000 μg/mL of BRB ‘Sembada Hitam’ extract can be applied in further treatment (Figure 2.).

Fig. 2: Extract of black rice ‘Sembada Hitam’ bran has no cytotoxic effect on NIH3T3 cells for 24 hours of incubation. Each bar represents different treatment. The value in each bar represent the mean ± SD (standard deviation), triplicates. The different letters in each bar represent significant difference of the value (p ≤ 0.05). BRB = black rice bran’s extract.
BRB’s extract treatment showed a slightly increasing cell viability, with no significant difference from the control group. 'Sembada Hitam' contains high anthocyanins (cyanidin-3-glucoside) 369.5 µg/100 g of rice seeds. Black rice also contains protein, fat, iron, tocopherol, zinc, and vitamin B such as thiamine and riboflavin, which were higher than brown rice and white rice.23 Black rice extract slightly increases fibroblast cell viability (RDFs), with no significantly different from the control group.22 The pericarp (outer layer) of black rice contains black pigments called anthocyanins, highly antioxidant activities that can prevent free radicals in the body.15

Fig. 3: Various concentrations of BRB’s extract significantly protect NIH3T3 cells against the cytotoxic effect of H₂O₂ exposure for 24 hours at low concentration but not at higher concentration. The combination of various concentrations of BRB’s extract for 24 hours on NIH3T3 cells with A. 50 µM H₂O₂ exposure, B. 100 µM H₂O₂ exposure, C. 150 µM H₂O₂ exposure, D. 200 µM H₂O₂ exposure, E. 300 µM of H₂O₂ exposure. Each bar represents different treatment. The value in each bar represents the mean ± SD (standard deviation), triplicates. The different letters in each bar represent significant difference of the value (p ≤ 0.05). BRB = black rice bran’s extract.
Cytoprotective of Black Rice ‘Sembada Hitam’ Bran’s Extract on NIH3T3 cells Induced by \( \text{H}_2\text{O}_2 \)

Based on the cytotoxicity result of BRB’s extract, it is known that the extract did not show a cytotoxic effect and maintained the viability of NIH3T3 cells. Further, we investigated whether the BRB’s extract has a cytoprotective effect against \( \text{H}_2\text{O}_2 \) exposure on NIH3T3 cells. The combination of \( \text{H}_2\text{O}_2 \) at various concentrations (50, 100, 150, 200, or 300 \( \mu \text{M} \)) and BRB’s extract at the concentration of 7.81 to 1000 \( \mu \text{g/mL} \) were exposed on NIH3T3 cells for 24 hours. Our results showed that 7.81 to 1000 \( \mu \text{g/mL} \) of BRB’s extract had a protective effect against 50 and 100 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) exposure. Moreover, BRB’s extract significantly increased cell viability at 500 and 1000 \( \mu \text{g/mL} \). However, higher concentrations of \( \text{H}_2\text{O}_2 \) exposure (150, 200, or 300 \( \mu \text{M} \)) combined with various concentrations of BRB’s extract were not effective to protect NIH3T3 cell viability. The combination of 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) exposure and various concentrations of BRB’s extract decreased more than 60% of NIH3T3 cell viability. Whereas, the combination of 300 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) exposure and various concentrations of BRB’s extract significantly caused a lethal condition on NIH3T3 cells (Figure 3.).

The protective effect of anthocyanin extract in black rice on rat dermal fibroblasts (RDF) increases cell viability against \( \text{H}_2\text{O}_2 \)-induced oxidative stress. \(^{24}\) Boonyanuphong and U Tobgay\(^{19}\) also reported that the antioxidant activity of black glutinous rice was able to prevent \( \text{H}_2\text{O}_2 \)-oxidative stress and reduce ROS levels on HT-29 cells. Anthocyanins regulate of PI3K/Akt and ERK1/2 expression as a cellular defense system against oxidative stress. Another study also reported the high antioxidant activity contained in the anthocyanin extract of black rice (\textit{Oryza sativa} L. Indica) (25 ppm of an extract with an antioxidant capacity of 824 ± 17.24 \( \mu \text{M} \)) was able to inhibit oxidation reactions that could protect and prevent DPPH radicals.\(^{11}\)

In normal physiological conditions, the intracellular \( \text{H}_2\text{O}_2 \) produced by mitochondria is around 10 \( \mu \text{M} \) or below while the rate of production in organs is around 50 \( \mu \text{M} \) per minute per gram. However, the susceptibility of plasma membrane permeability to extracellular \( \text{H}_2\text{O}_2 \) can disrupt ROS homeostasis and modulate redox signaling pathways, so that \( \text{H}_2\text{O}_2 \) can diffuse into cells and be decomposed easily by intracellular antioxidants that triggers oxidative stress.\(^{1,25}\) Induction of \( \text{H}_2\text{O}_2 \) at the concentrations of 150 and 200 \( \mu \text{M} \) on hADMSCs cells caused DNA damage.\(^{26}\) Another study also reported cytoplasmic condensation and increased intracellular gap on human lens epithelial cells (HLE) shown at the concentration of 200 \( \mu \text{mol/L} \) \( \text{H}_2\text{O}_2 \) exposure represented apoptosis cells.\(^{27}\)

Anti-apoptotic of Black Rice ‘Sembada Hitam’ Bran’s Extract on NIH3T3 Cells Induced by \( \text{H}_2\text{O}_2 \)

The features of \( \text{H}_2\text{O}_2 \)-induced apoptotic cells were detected using a double-staining method using AO-PI which detected green nuclei as a living cell and red nuclei as an apoptotic cell. Treatment of BRB’s extract at the concentration of 62.5 \( \mu \text{g/mL} \) was shown effective in suppressing apoptotic cells caused by \( \text{H}_2\text{O}_2 \) exposures at 100 and 200 \( \mu \text{M} \). Whereas, 250 \( \mu \text{g/mL} \) of BRB’s extract was effective in suppressing cell apoptosis after \( \text{H}_2\text{O}_2 \) exposure for 24 hours at the concentration of 100 \( \mu \text{M} \). In contrast, 250 \( \mu \text{g/mL} \) of BRB’s extract was not effective in suppressing cell apoptosis after \( \text{H}_2\text{O}_2 \) exposure for 24 hours at 200 \( \mu \text{M} \). AO/PI double staining showed a change in the color and morphology of apoptotic cells. Control cells display a bright green color and intact nuclear structure, indicating healthy cells, whereas red stained cells indicate the presence of apoptotic cells. Our results showed that \( \text{H}_2\text{O}_2 \) induced cell death through an apoptotic pathway dose-dependent manner. Figure 4. shows a shift from viable cells to apoptotic cells represented red nuclei and live cells represented green nuclei. At the combination concentration of 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) and 250 \( \mu \text{g/mL} \) of BRB’s extract showed that the red fluorescence in the cells significantly increased, indicates the number of cell death and shrinkage (Figure 4.). We predicted that the antioxidant activity of BRB’s extract has an anti-apoptotic role in \( \text{H}_2\text{O}_2 \)-induced cell death.

Anthocyanin form extract of black rice was effective in suppressing caspase-3 from \( \text{H}_2\text{O}_2 \) and induced apoptosis in RDF cells.\(^{21,24}\) Cytochrome P450 enzyme (CYP) can convert toxic metabolites into ROS, such as \( \text{H}_2\text{O}_2 \) which can trigger oxidative stress to cause biological processes such as apoptosis. Cells that collectively reduce the oxidative state through an antioxidant system consisting of enzymatic antioxidants, can manipulate ROS levels by regulating gene expression and related signaling pathways to maintain redox balance and integrity of cellular components.\(^{28}\) Antioxidant
enzymes protect cell damage by decreasing the expression of pro-apoptotic proteins. In addition, Nrf2 regulation increases CAT expression which converts $H_2O_2$ produced by SOD into $H_2O$ and $O_2$ to inhibit oxidation on HDF cells and causes anti-apoptotic conditions.

Fig. 4: Apoptotic feature of NIH3T3 cells pretreated with combination of BRB’s extract and $H_2O_2$.
A. Combination exposure of the black rice bran’s extract and 100 μM $H_2O_2$ on NIH3T3 cells did not cause cell death via apoptotic pathway. The picture represents AO/PI staining of NIH3T3 cells were viewed under a confocal microscope at 24 hours. B. Control cell C. 62.5 μg/mL of BRB’s extract and 100 μM $H_2O_2$ D. 62.5 μg/mL of BRB’s extract and 200 μM $H_2O_2$ E. 250 μg/mL of BRB’s extract and 100 μM $H_2O_2$ F. 250 μg/mL of BRB’s extract and 200 μM $H_2O_2$. Each bar represents different treatment. The value in each bar represent the mean ± SD (standard deviation), triplicates. The different letters in each bar represents significant difference of the value ($p \leq 0.05$). BRB = black rice bran’s extract.

Red arrow heads indicated living cell and yellow arrow heads indicated apoptotic cell.
Our study showed that the BRB’s extract has antioxidant activity by protecting and maintaining cell viability from H₂O₂ stress at the concentrations of 100 μM or lower. Whereas, at the high concentrations of more than 100 μM, the BRB’s extract at the concentration of 250 μg/mL was not able to protect cell viability. It indicates that the higher concentration of the extract, it may has a prooxidant effect which causes a reduction in cell viability. Various studies reported concentration-dependent antioxidant activity and prooxidants in natural antioxidants. The antioxidant activity of low concentrations of Trolox (2.5-15 μM) significantly decreases ROS production. However, the effect of prooxidants at the higher concentrations (20-160 μM) induces apoptosis. The Sinlek rice bran extract had antioxidant activity at the concentration of 0.005-0.5 mg/mL and prooxidant activity at the higher concentrations (5-7.5 mg/mL) against 1 mM H₂O₂ induced oxidative stress on Caco-2 cells. Whereas, in Riceberry bran extract, at the concentrations of 15 and 17.5 mg/mL had antioxidant activity and at the concentration of 20 mg/mL prooxidant activity eliminated the protective effect. Vitamin C, vitamin E, carotenoids, and polyphenols have an important role in the endogenous antioxidant defense system. Antioxidants in physiological dose ranges are able to protect against oxidative stress and excessive concentrations cause damage through prooxidant effects. Moreover, prooxidant activity also catalyzed by Fe and Cu produces free radicals to mutagenesis in biological systems.

Black Rice ‘Sembada Hitam’ Bran’s Extract Protects Cell Growth of NIH3T3 Cells Induced by H₂O₂

Protective effect of the BRB’s extract on cell survival against H₂O₂ exposure were assessed through cell growth assays on the 3rd and 5th day. The results showed that the cells were able to proliferate constantly after H₂O₂ exposure for 24 hours together with BRB’s extract treatment. The BRB’s extract at the concentration of 62.5 μg/mL significantly increased cell proliferation after 100 μM of H₂O₂ exposure for 24 hours. However, the cells which exposed with 200 μM of H₂O₂ started to stop their proliferation after 3 days culture. Interestingly, the treatment of BRB’s extract at the concentration of 250 μg/mL showed lower cells proliferation compared to that of 62.5 μg/mL against H₂O₂ exposure at the concentration of 100 and 200 μM (Figure 5.). Based on these results, we suggested that the BRB’s extract can prolong the life span of NIH3T3 cells up to 5 days after 24 hours exposure of H₂O₂ on the first day.
Fig. 5: The black rice bran’s extract increases cell proliferation of NIH3T3 cells after H$_2$O$_2$ exposure for 24 hours at the first day. A. Pretreatment of 62.5 μg/mL BRB’s extract B. Pretreatment of 250 μg/mL BRB’s extract. Each bar represents different treatment. The value in each bar represents the mean ± SD (standard deviation), duplicates. The different letters in each bar represents significant difference of the value (p ≤ 0.05). BRB = black rice bran’s extract.

All kinds of oxidative damages reduce the replicative capacity and shorten the lifespan of the cells and organisms can be repaired by antioxidants. Natural and synthetic antioxidants can extend replicative life span through the activity of signaling pathways and proteasome activation. The antioxidant effect of polyphenolic compounds in quercetin has been shown to protect IPEC-J2 cells from H$_2$O$_2$-induced apoptosis and 5 μg/mL of quercetin promote cell division. Pretreatment of paeoniflorin (root extracted of Paeonia lactiflora Pall.) for 24 hours could attenuate apoptosis in PIG1 and PIG3V cells, but not effective for 48 hours treatment. Potency of paeoniflorin to counteract H$_2$O$_2$-induced oxidative damage on PIG1 and PIG3V cells were predicted by JNK and Nrf2/HO-1 pathway. Moreover, H$_2$O$_2$ induced premature senescence and could be improved by over-expression of NAMPT on MEF cells.

Conclusion
The BRB’s extract has potency to suppress apoptosis and maintain cell viability as well as cell growth against H$_2$O$_2$-induced oxidative stress. These results can be considered for application as a nutraceutical in chronic diseases mediated by oxidative stress and anti-aging cosmetic. The senescence regulation such as cell cycle arrest and DNA repair are required to further understand the cellular response.

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Conflict of Interest
This article is the authors original work and is not under consideration for publication elsewhere. The corresponding author has full responsibility for the progress revisions until final approval of the manuscript. The authors declare no conflict of interest, or any personal business interest, affiliation, or activity with any entity.

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