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Impact of Volcanic-Derived Polycyclic Aromatic Hydrocarbons on Apoptosis, Necrosis and p53 Expression in Hawaiian Green Turtle Lung Cells

Renhe Bai

Hawaii Preparatory Academy, HI 96743, United States
rbai@hpa.edu

Abstract:

Polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants that are known for their cytotoxic, mutagenic, and carcinogenic effects on human health. However, compared to their known effects on human health, their impact on marine species remains relatively unexplored. In this study, we investigate the cellular toxicity of volcanic-derived PAHs on green turtle (Chelonia mydas) lung cells, particularly under the broader concerns of the recent Kīlauea volcanic eruption on Hawaii's Big Island. In the experiment, isolated green turtle lung cells are treated with various concentrations of PAHs and assessed by cell viability, apoptosis, and p53 expression. Under analysis and discussion, the literature review highlights the potential adverse effect of volcanic pollutants to marine life and also underscores the need for conservation strategies to protect the green turtle and other vulnerable species.

Keywords:-volcanic-derived PAHs; green turtle lung cells; apoptosis; p53 expression

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are classified as a type of organic compound in chemistry and are comprised of multiple aromatic rings [1]. They are common environmental pollutants that are widely distributed in water, soil, and, particularly, in the atmosphere. With respect to their formation, PAHs are mainly from intermediate products of incomplete combustion of organic materials such as tobacco, timber, and fossil fuels, which suggests that combustion reaction is one of the major sources of their widespread distribution. Accordingly, forest fires and volcanic eruptions become one of their major causes [1].

In previous research, there has been extensive evidence showing that PAHs have significant adverse effects on human health due to their toxic properties. That is, when human cells are exposed to PAHs, it can cause mutations in the TP53 gene, which plays a critical role in modulating cell cycle and abnormal apoptosis, which thereby can increase the risk of can-

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cer development. [1] Other studies have shown that direct exposure to PAHs through inhalation can result in apoptosis and necrosis in human lung cells, which leads to tissue damage and increases the incidence of lung disease in humans, such as lung cancer. [2] Moreover, recent research suggests that PAHs can induce oxidative stress and trigger inflammatory responses, causing damage to mitochondrial DNA and essential structural proteins, which highlights their detrimental impacts on cells at the microscopic level. [3]

Despite the well-documented effects of PAHs on human health, there has been a lack of attention on their impact on other species. Since the recent eruption of Kīlauea volcano on June 3, 2024, which significantly elevated Hawaii Big Island atmospheric PAHs level, public concerns about the potential negative impacts of PAHs on local ecosystems have been raised, particularly of the health of marine life that inhabit the region; however, the effects of PAHs on these important marine species remains largely unexplored.

Sea turtles, particularly green turtles, are integral components of local marine ecosystems and can serve as indicators of environmental health because of their longevity, mobility, and position in the food chain [4] [5]. These turtles play a significant role in keeping the health of coral reefs and seagrass beds, which is vital to the local marine ecosystem. Their grazing, for instance, helps keep seagrass beds healthy and productive, benefiting other marine species [6]. In specific, Hawaii green turtles, or honu, are culturally significant and have been a part of Hawaiian traditions and heritage for centuries; however, they are considered a threatened species under the Endangered Species Act. Their population, while recovering, still faces numerous threats from habitat loss, climate change, and pollution, making them a vulnerable species [7]. From previous papers, it is shown that green turtles are especially susceptible to environmental contaminants such as PAHs due to their bimodal respiratory system, which inevitably increases the direct contact between sea turtles and their environment [7]. Therefore, by studying the effects of PAHs on these turtles, we can gain valuable insights into the broader impacts of volcanic-derived pollutants on marine ecosystems and develop more effective conservation strategies.

Apoptosis refers to programmed cell death, and it is one of the indicators to measure and quantify the detrimental effects of PAHs on sea turtle lung cells in the experiment. It is a tightly regulated process that enables cells to die in a controlled manner, which can be measured by MTT assay [8]. In the process of apoptosis, a series of biochemical events are involved, including the activation of caspases, the release of cytochrome c from mitochondria, and the subsequent formation of the apoptosome complex [9]. During these processes, characteristic cellular changes will be directly or indirectly caused, which include cell shrinkage, chromatin condensation, DNA fragmentation, and membrane blebbing, all of which will ultimately lead cells to undergo a programmed death [10]. In contrast, necrosis is another form of uncontrolled cell death. Different from apoptosis, it may result from acute cellular injury, such as from toxins, trauma, or infection [8]. In the paper, apoptosis, and necrosis are both used to measure and assess the impact of PAHs on cellular health.

The p53 signaling pathway is a regulatory network that is used to indicate cell apoptosis in the experiment, which is encompassed by the p53 protein, a crucial tumor suppressor protein that is encoded by the tumor protein p53 (TP53, or p53) gene. In vivo, p53 pathway plays a crucial role in regulating cell cycle and maintaining genomic stability, which acts as a transcription factor that responds to different kinds of cellular stress signals such as DNA damage, oxidative stress, oncogene activation, etc. Therefore, the p53 pathway generally serves as a crucial regulator of apoptosis and necrosis in order to respond to cellular stress and DNA damage [11].

The purpose of the study is to address the gap in knowledge regarding the effects of PAHs on green turtle lung cells, which mainly focuses on examining the impact of PAHs on cell apoptosis, p53 signal pathway response, and subsequent cellular outcomes. The research will improve the understanding of the mechanism of PAHs on living cells and how environmental toxins affect these marine species.

In light of the established toxicity of PAHs on human lung cells by prior research, it is predicted that direct exposure to increasing concentrations and treatment durations with volcanic-derived polyaromatic hydrocarbons (PAHs) through inhalation can induce apoptosis and necrosis in isolated turtle lung cells and increase p53 expression.

2. Methods and Materials

2.1 Cell Culture

The primary isolated green turtle (Chelonia mydas) lung cells will be obtained from the Big Island NOAA Fisheries organization. The cells will be cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 1% glutamine at 37°C in a humidified incubator with 5% CO_2 [12].

2.2 PAH Preparation

PAHs such as benzo[a]pyrene and anthracene will be produced by organic material under a combustion reaction with controlled conditions. Concentration and purification will be achieved through rotary evaporation and chromatographic techniques. Purified synthesized PAHs will be dissolved in DMSO to prepare a stock solution, which will be stored at -20°C. Working solutions will be prepared fresh before each experiment by diluting the stock solution in PBS to achieve the desired concentrations.

2.3 Experimental Design

Cells will be divided into several groups for treatment. In the experimental group, green turtle lung cells will be treated with varying concentrations of PAHs, 1 μM, 10 μM, 50 μM, 100 μM respectively for different durations of 0 hours, 6 hours, 12 hours, 24 hours, and 48 hours. In the negative control group, green turtle lung cells will be treated with PBS/DMSO. In the positive control group, green turtle lung cells will be treated with 100 μM Hydrogen Peroxide (H_2O_2). Cell viability will be measured by MTT Assay, apoptosis and necrosis will be measured by Flow Cytometry, and the level of p53 expression by western blot.

2.4 Cell Viability Measured by MTT Assay

Seed green turtle lung cells in a 96-well plate at a density of 1*10^4 cells per well in 100 μL of complete DMEM. Incubate the cells overnight at 37°C with 5% CO_2 to allow them to attach. Treat cells with PAHs solutions with concentrations of 1 μM, 10 μM, 50 μM, 100 μM into each line respectively. Include negative control wells with PBS/DMSO only and positive control wells with 100 μM H_2O_2. Incubate for 0, 6, 12, 24, and 48 hours. Then add 20 μL of MTT solution (5 mg/mL in PBS) to each well. Incubate for 4 hours at 37°C. Carefully remove the medium and add 150 μL of DMSO to each well to dissolve the formazan crystals. Measure the absorbance at 570 nm with a microplate reader. [13] Cell viability in percentage will be calculated by the direct proportion of the absorbance of treated cells to the absorbance of control cells times a hundred percent.

2.5 Apoptosis and necrotic measured by Annexin V/PI FACS

Seed green turtle lung cells in a 6-well plate at a density of 2*10^5 cells per well in 2 mL of complete DMEM. Incubate overnight at 37°C with 5% CO_2. [14] Replace the medium with PAHs solutions in PBS/DMSO at 1 μM, 10 μM, 50 μM, 100 μM respectively. Include control and positive control wells as mentioned above. Incubate for 0, 6, 12, 24, and 48 hours. Harvest the cells by trypsinization and wash them twice with cold PBS.

Resuspend cells in 1X binding buffer at a concentration of 1x10^6 cells/mL. Transfer 100 μL of the cell suspension to a new tube. Add 5 μL of Annexin V-FITC and 5 μL of propidium iodide (PI). Incubate for 15 minutes at room temperature in the dark. After staining the cells, add 400 μL of 1X binding buffer to each tube and analyze the stained cells using a flow cytometer within 1 hour. Gate the cells to distinguish live, early apoptotic, and late apoptotic/necrotic populations based on FITC and PI staining.

The percentage of cells in each population (live, early apoptotic, and necrotic) will be quantified with FlowJo [14].

2.6 p53 expression measured by Western blot

Seed green turtle lung cells in a 6-well plate at a density of 2*10^5 cells per well in 2 mL of complete DMEM. Incubate overnight at 37°C with 5% CO_2. Replace the medium with PAHs solutions in PBS/DMSO at 1 μM, 10 μM, 50 μM, 100 μM respectively. Include control and positive control wells as mentioned above. Incubate for 0, 6, 12, 24, and 48 hours. Wash the cells twice with cold PBS and lyse them in a RIPA buffer containing protease and phosphatase inhibitors. Collect the lysates and centrifuge at 14,000 rpm for 15 minutes at 4°C. Collect the supernatant and determine protein concentration using a BCA protein assay kit [15]. After protein extraction, load equal amounts of protein (30 μg) onto SDS-PAGE gels and perform electrophoresis and transfer the proteins to a PVDF membrane. Block the membrane with 5% non-fat milk in TBST for 1 hour at room temperature [15]. Incubate the membrane overnight at 4°C with primary antibodies against p53 (1:1000) and β-actin (1:5000). Wash the membrane with TBST and incubate with HRP-conjugated secondary antibodies (1:2000) for 1 hour at room temperature. Wash the membrane and detect protein bands using ECL detection reagent. Visualize and quantify the bands using a chemiluminescence imaging system. Normalize p53 expression levels to β-actin as a loading control [15]. The relative expression of p53 in treated and control groups will be compared with densitometry software, ImageJ.

2.7 Statistical Methods

Each experiment will be repeated three times, and the data will be reported as mean ± standard deviation. Statistical analysis will be performed using GraphPad Prism 9.0. An unpaired Student's t-test will be used for analyzing the MTT cell viability assay results, comparing the control and PAH-treated groups; for the data obtained from

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Annexin V/PI flow cytometry and Western blot analysis of p53 expression, one-way ANOVA will be employed to assess differences among multiple treatment groups, followed by Tukey's post-hoc test for specific group comparisons; western blot images will be processed with Image Studio™ Software 5.5.4 to quantitatively analyze band intensity. A significance level of $p<0.05$ will be considered statistically significant.

3. Results

Table 1 The combination of possible results

Combination of possible results (CR)	PAHs decrease viability by MTT?	PAHs increases apoptosis by Annexin V/PI FACS?	PAHs increases p53 by western blot?	Support of hypothesis
CR 1	+	+	+	Yes
CR 2	+	+	-	Partial
CR 3	+	-	+	Partial
CR 4	-	+	+	Partial
CR 5	+	-	-	Partial
CR 6	-	+	-	Partial
CR 7	-	-	+	Partial
CR 8	-	-	-	No

The "+" sign means that the heading phenomenon observed is similar to the positive control and opposite to the negative control in the experiments and is statistically significant. The "-" sign means that the phenomenon observed is neither similar to the positive control group nor opposite to the negative control or is not statistically significant.

CR 1: After exposure to PAHs, cell viability statistically decreases compared to the negative control group measured by MTT assay; Cell apoptosis increases compared to the negative control group measured by Annexin V/PI FACS; p53 expression increases compared to the negative control group measured by western blot.

CR 2: After exposure to PAHs, cell viability statistically decreases compared to the negative control group measured by MTT assay; Cell apoptosis increases compared to the negative control group measured by Annexin V/PI FACS; p53 expression does not increase compared to the negative control group measured by western blot.

CR 3: After exposure to PAHs, cell viability decreases, measured by MTT assay; Cell apoptosis does not increase, measured by Annexin V/PI FACS; p53 expression increases, measured by western blot.

CR 4: After exposure to PAHs, cell viability does not decrease, measured by MTT assay; Cell apoptosis increases, measured by Annexin V/PI FACS; p53 expression increases, measured by western blot.

CR 5: After exposure to f PAHs, cell viability decreases measured by MTT assay; Cell apoptosis does not increase measured by Annexin V/PI FACS; p53 expression does not increase measured by western blot.

CR 6: After exposure to PAHs, cell viability does not decrease, measured by MTT assay; Cell apoptosis increases, measured by Annexin V/PI FACS; p53 expression does not increase, measured by western blot.

CR 7: After exposure to PAHs, cell viability does not decrease, measured by MTT assay; Cell apoptosis does not increase, measured by Annexin V/PI FACS; p53 expression increases, measured by western blot.

CR 8: After exposure to PAHs, cell viability does not decrease by MTT assay; Cell apoptosis does not increase measured by Annexin V/PI FACS; p53 expression does not increase measured by western blot.

4. Discussion

The purpose of the experiment is to investigate the potential adverse effects of PAHs on green turtle's lung cells, and in the research, cell viability, apoptosis, and p53 expression are measured to evaluate these effects. There are eight combinations of possible results that come from the experiment and will be discussed below in detail.

The results from CR 1 strongly support the hypothesis, which suggests that PAHs may exert a multifaceted toxic impact on green turtle lung cells, which is similar to their established effects on human lung cells in previous research. In this case, the increased p53 expression indicates that oxidative stress and DNA damage are induced by PAHs, thus causing cell apoptosis and necrosis. This result corroborates previous findings by Stading et al. on human lung cells, which suggest that PAHs potentially have universal mechanisms of toxicity across different species. [2]

From results in CR 2, PAHs show their effects on decreasing cell viability and increasing apoptosis, but without a concomitant increase in p53 expression. This result partially supports the hypothesis, which suggests that there may be an alternative apoptotic pathway that responds to cell apoptosis caused by PAHs. This phenomenon, which lacks a p53 elevation, could imply that PAHs might induce apoptosis through p53-independent mechanisms, which is similar to Zheng et al. 's finding in retinal pigment epithelium cells under a specific stress condition. [16] Future research should investigate these alternative pathways to better understand the apoptotic mechanism of green turtle lung cells.

In CR 3, PAHs decrease cell viability and increase p53 expression but do not significantly induce apoptosis that measured by Annexin V/PI FACS. This result partially supports the hypothesis, which indicates that there may be a possible early or sub-lethal stress response. In this case, the cells might be undergoing cell cycle arrest mediated by p53, and then activate their repair mechanisms before they fully commit to apoptosis. This observation aligns with findings by Venkatachalam et al. (2017) in human bronchial cells. [17] Alternatively, another explanation may suggest that the cells might undergo other ways of systematical death, such as necrosis, and due to this, cell apoptosis is not detected in the experiment. According to this case, future studies should focus on exploring the duration and dose-dependence of the cell apoptosis response and investigating alternative ways of cell death.

In CR 4, the result shows that the stress of PAHs increases cell apoptosis and p53 expression, however, without causing a decrease in cell viability, which partially supports the hypothesis. This result may suggest a threshold effect, where PAHs initiate stress responses that could eventually lead to cell death if exposure persists or increases. The results' partial support points to a time or dose-dependent aspect of PAH toxicity, supported by research from Bommuraj et al., where initial cellular stress responses precede observable declines in viability. [18] Future experiments should vary exposure durations and concentrations to determine the precise conditions under which PAH toxicity becomes lethal.

In CR 5, CR 6, and CR 7, results show significant effects in only one parameter, decreasing cell viability, increasing apoptosis, or increasing p53 expression, which provides limited support for the hypothesis. In CR 5, the reduction in cell viability without increased apoptosis or p53 expression might indicate non-apoptotic cell death pathways or sub-toxic levels of PAHs that impair metabolic functions. In CR 6, increased apoptosis without corresponding changes in cell viability or p53 levels may reflect acute but transient stress responses that are insufficient to cause widespread cell death or significant p53 activation. In CR 7, the increase in p53 expression without corresponding changes in cell viability or apoptosis suggests that PAH exposure might trigger a stress response in green turtle lung cells without causing immediate cell death. This indicates that p53 activation could be part of an early or protective cellular response to PAHs, potentially initiating cell cycle arrest and repair mechanisms rather than leading directly to apoptosis or necrosis. Future research should focus on investigating non-apoptotic pathways, delineating the timeline and specific conditions, and understanding the long-term effects of sustained p53 activation.

In CR 8, all parameters do not show significant changes, which suggests that the PAH concentrations or exposure durations failed to reach the thresholds that are required to elicit detectable cellular responses. This result fully contradicts the hypothesis, which indicates it may require optimizing conditions to ensure that the applied stress is sufficient to trigger measurable effects in future experiments.

In addition, the results of this study will be contingent upon the varying concentrations of PAHs and treatment durations. If higher concentrations of PAHs result in significantly decreased cell viability and increased apoptosis and necrosis, it would corroborate the hypothesis that volcanic-derived PAHs are highly toxic to green turtle lung cells. Conversely, if lower concentrations exhibit similar toxic effects to high-dose treatment groups, it would suggest that PAHs are exceptionally toxic, which indicates that even minor volcanic eruptions could have severe biological impacts. Regarding treatment durations, if extended treatment durations result in pronounced cell damage, it would imply that chronic exposure is particularly detrimental, whereas if shorter durations yield comparable results, it would indicate that PAHs may have rapid toxicity onset. If there are no significant changes observed at any concentration or duration, it will challenge the hypothesis, suggesting green turtle lung cells might possess the resilience to PAHs or that other factors are involved in toxicity. For future experiments, a broader range of PAH concentrations and durations should be explored, which may include additional biomarkers of oxidative stress and DNA damage. Additionally, future research could investigate the combined effects of PAHs with other environmental stressors to better simulate real-world conditions and elucidate the mechanisms underlying PAH toxicity in marine organisms.

5. Conclusion

This study investigates the effects of volcanic-derived polyaromatic hydrocarbons (PAHs) on green turtle lung cells,

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focusing on cell viability, apoptosis and necrosis of cells, and p53 expression. Multiple combinations of possible results highlight the biological toxicity of PAH and the need for further research to elucidate the underlying mechanisms and long-term impacts on marine life. As efforts in the paper, assessing the environmental health risks posed by volcanic activity such as PAHs has shown its necessity for the development of conservation strategies for green turtles and other vulnerable species in the aquatic ecosystem.

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