

NEUROBLASTOMA CELL-LINE TOXICITY DUE TO INCREASED ALZHEIMER'S-RELATED PROTEINS AND CELL DEATH SUBSEQUENT TO HIGH-DOSE PALMITIC ACID EXPOSURE

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Abstract:

Obesity caused by high-fat diet (HFD) consumption is one of the risk factors for the development of Alzheimer's disease (AD). Previous studies have reported that saturated fatty acids from an HFD, particularly palmitic acid (PA), promotes Alzheimer's pathologies, neuroinflammation and cognitive impairment. The pathological hallmarks of AD are characterized by the accumulation of amyloid- β ($A\beta$) peptides and phosphorylated Tau (pTau) proteins. However, the expression of $A\beta$ peptides, and Tau proteins levels, as well as the viability of cell in neuronal cells induced by PA remain unclear. Therefore, this study aims to determine the effect of high concentrations of PA on cell viability and Alzheimer's-related proteins of neuronal cells. In this study, SH-SY5Y was used as a neuronal cell model in order to investigate the effect of PA at a concentration of 400 and 800 μ M. The results showed that neuronal cell exposed to PA at doses of 400 and 800 μ M for 24 and 48 hours had decreased cell viability in a dose-dependent manner. In addition, receiving PA for 48 hours induced $A\beta$ peptides and p-Tau proteins production. Our study suggests that high concentration of PA exposure can lead to increased risk of the development of Alzheimer's pathologies and neuronal cell death.

Keywords: Palmitic acid; Alzheimer's disease; Neuronal cell; Amyloid beta; Tau

Introduction

According to previous studies, long-term high-fat diet (HFD) consumption is one of the key factors in obesity around the world. Obesity enhances the impairment of learning and memory performance and the development of Alzheimer's-like pathophysiological changes in the brains (Granhölm *et al.*, 2008). Palmitic acid (PA) is the most common form of saturated fatty acid (FA) in HFD. PA has been found to be an abundant saturated FA existing in the human body and is closely linked to causing lipotoxicity (Plötz *et al.*, 2016). In neuronal cells, PA is known to induce endoplasmic reticulum stress, impaired cell proliferation, abnormal cell differentiation, and resulting in cellular apoptosis (Park *et al.*, 2011; Wang *et al.*, 2014).

Alzheimer's disease (AD), which is the most common neurodegenerative disease, is characterized by cognitive decline, memory dysfunction, and behavioral impairment. In 2015, 46.8 million people worldwide were living with dementia and the number is expected to reach

75 million in 2030 and 131.5 million in 2050 (Martin *et al.*, 2016). AD is caused by brain cell death, and this brain cell death happens progressively over time. An abundance of intracellular and extracellular aggregation was found in affected brain regions. Neuronal intracellular aggregates called neurofibrillary tangles consist of hyperphosphorylated Tau proteins in the form of insoluble paired helical filaments (Lee *et al.*, 2001). The abnormal hyperphosphorylation of Tau results in the loss of Tau function in promoting microtubules stabilization, and eventually lead to cell death (Iqbal *et al.*, 2005). Neuronal extracellular deposits called amyloid plaques consist of amyloid β ($A\beta$) peptides in the form of insoluble amyloid fibrils. The abnormal regulation of amyloid precursor protein (APP) causes the accumulation of $A\beta$ resulting in the development of systemic inflammation. Excessive $A\beta$ plaques and neurofibrillary tangles are the primary causes of AD. However, the underlying mechanism of PA-induced neuronal cell death which is associated with the upregulation of Alzheimer's-related proteins has not been thoroughly investigated. Therefore, the purpose of this study is to investigate the effects of high concentrations of PA on cell viability and the expression of Alzheimer's-related proteins. The concentration of PA in this study was referred to previous study that SH-SY5Y was died after exposed to 200-500 μM of PA for 24 and 48 hrs (Hsiao *et al.*, 2014). Accordingly, we chose PA at the concentration of 400 and 800 μM to investigate the effect of high dose of PA. We hypothesized that PA is involved in the progression of AD and is related to neuronal toxicity and neuronal cell death.

Materials and Methods

Experimental Protocol

SH-SY5Y cells were divided into 3 groups according to the PA treatment protocol, each group consisted of 4 cell samples. 1) The control group: SH-SY5Y cells receiving a vehicle (0.02 mM, NaOH), 2) the PA 400 μM group: SH-SY5Y cells receiving PA at a concentration of 400 μM , 3) the PA 800 μM group: SH-SY5Y cells receiving PA at a concentration of 800 μM . To determine cell viability, SH-SY5Y cells were exposed to PA for 4, 24 and 48 hrs and viability was determined by the alamarBlue assay. To investigate the expression of Alzheimer's-related proteins including of $A\beta$, APP, p-Tau and Tau, SH-SY5Y cells were exposed to PA for 48 hrs then a western blot analysis was performed. The diagrams of experiment are illustrated in Figure 1.

Cell Culture

This study used the SH-SY5Y cell line (human neuroblastoma cell line) as an *in vitro* model due to its wide use in neuronal function and differentiation research. SH-SY5Y cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). SHSY5Y cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)/Ham's Nutrient Mixture F12 with glutamax (1:1; v/v), sodium bicarbonate, 10% fetal bovine serum and 1% penicillin-streptomycin and incubate at 37 °C in a 5% CO₂ incubator. Fresh medium was changed every 2-3 days.

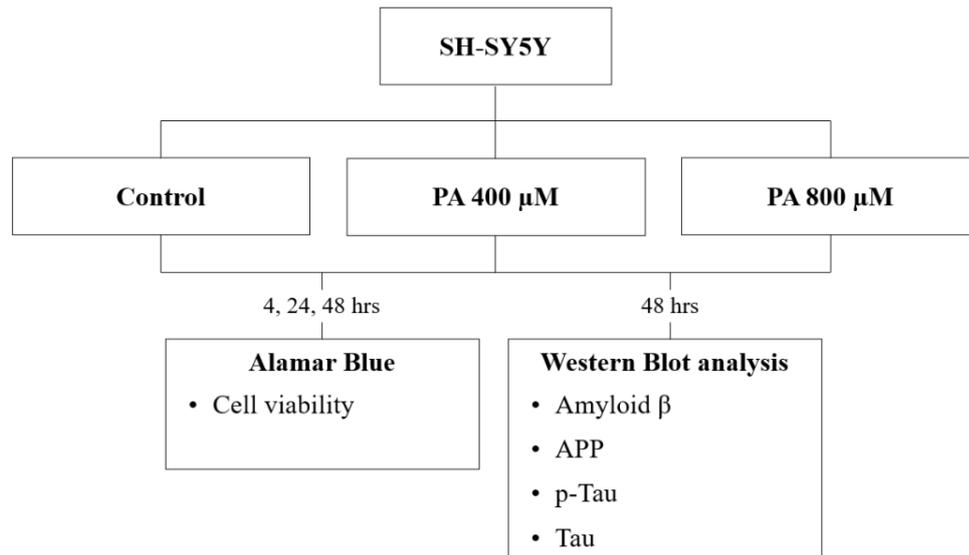


Figure 1: Diagram of experimental protocol

Preparation of Palmitic Acid Solution

First, the stock of 40 mM PA solution was prepared by dissolving 10.26 mg of PA in 0.01 M Sodium hydroxide (NaOH) in a dry bath at 80 °C for 15 minutes. The PA was purchased from Sigma-Aldrich (Dorset, UK). In addition, 1% free fatty acid (FFA)-free BSA was dissolved in DMEM at 37°C. After that, the PA was mixed with FFA-free BSA by adding different volumes of PA stock solution to reach final concentrations of 16 and 32 mM of PA for PA200 and PA400 groups, respectively. The mixtures were incubated in water bath at 37 °C for 30 min before being treated. The vehicle control consisted of 1% of FFA-free BSA media with 0.02 mM NaOH.

Cell Viability Assessment by AlamarBlue Assay

SH-SY5Y cells were plated in 96-well plate with 1×10^4 cells/well then cultured in DMEM at 37 °C in a 5% CO₂ incubator overnight. After that, cells were treated with vehicle and PA at concentration of 16 and 32 mM to reach the final concentration of PA in the control, PA 400 μM and PA 800 μM groups, respectively. To determine cell viability, alamarBlue reagent was then added in each well. alamarBlue reagent is composed of Resazurin dye which is used in redox based colorimetric assays to determine cellular metabolic reduction. Living cells change Resazurin from blue and non-fluorescent to pink and highly fluorescent. After 4, 24 and 48 hrs of PA exposure, the absorbance was measured at 570 nm and 590 nm. The intensity of fluorescence produced is proportional to the number of living cells. To calculate the percentage of cell viability, the absorbance of control group was used as a reference number of cells. The absorbance of PA 400 and 800 μM group was divided by the absorbance of the control group at the same time point and then multiplied by 100 as shown in this following equation.

$$\%Cell\ viability = \frac{Absorbance\ of\ PA\ group\ at\ each\ time\ point}{Absorbance\ of\ control\ group\ at\ the\ same\ time\ point} \times 100$$

Alzheimer's-Related Protein Measurement by Western Blot Analysis

SH-SY5Y cells were plated in 6-well plate with 2.5×10^5 cells/well overnight. Then, cells were treated with vehicle and 16 and 32 mM of PA to reach final concentration of PA in the control, PA 400 μ M and PA 800 μ M group, respectively, for 48 hrs. Next, SH-SY5Y cells were lysed with radioimmunoprecipitation assay (RIPA) buffer, 1% Triton X-100 and 1% protease inhibitor and measured the concentration of total protein by Bicinchoninic acid (BCA) assay. After that, the prepared proteins were separated by electrophoresis method called SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) and transferred onto nitrocellulose membranes. Membranes were blocked for one hour with 5% nonfat dry milk or 5% BSA, according to the manufacturer recommendation, in Tris-buffer saline (pH 7.4) containing 0.1% Tween 20. Next, the membranes were probed with the primary antibodies (1:1000) against Alzheimer's-related proteins including A β (Santa Cruz Biotechnology, USA), APP, p-Tau, Tau, (Cell Signaling Technology, USA), and a loading control (GAPDH) (Abcam, USA). After that, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) at room temperature for 1 hour. Finally, the membranes were visualized by Clarity™ Western ECL Blotting Substrate (Bio-Rad Laboratories Ltd.) and scanned by ChemiDoc™ Touch Gel Imaging System. Image J (NIH image) analysis software was used to analyze the intensity of western blotting images.

Statistical Analysis

The data for each experiment was expressed as mean \pm standard error of the mean. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test were performed on the expression of Alzheimer's-related proteins. Two-way ANOVA analysis followed by Fisher's LSD comparisons test was used for the cell viability assessment. GraphPad Prism software was used in this study. *P*-value < 0.05 was considered statistically significant.

Results

The Effects of PA on SH-SY5Y Cell Viability

The result of the cell viability assessment from alamarBlue assay is shown in Figure 2. We found that SH-SY5Y cells exposed to PA in the concentration of 400 and 800 μ M had significantly decreased in the percentage of cell viability. Interestingly, the decrease of cell viability was proportional to the increase PA concentration. Cell viability of SH-SY5Y cells was significantly decreased, when exposed to 400 and 800 μ M of PA for 24 and 48 hrs when compared to that condition for 4 hrs. However, the statistical analysis did not show the differences in cell viability between 24 and 48 hrs of treatment. These findings suggested that the exposure of PA at both concentrations of 400 and 800 μ M for 4, 24 and 48 hrs caused neuronal toxicity and cell death to SH-SY5Y cell line. The images of SH-SY5Y cell line after exposure to 400 and 800 μ M of PA for 24 and 48 hrs are shown in Figure 3.

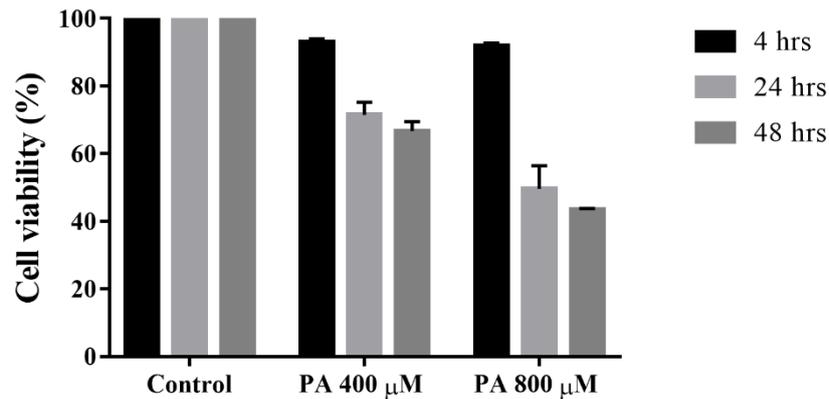


Figure 2: Cell viability of SH-SY5Y after exposed to 400 and 800 μ M of PA for 4, 24 and 48 hrs. N = 4 per group in each time point. The values are presented as mean \pm SEM. * $p < 0.0001$ compared with control group at the same time point, † $p < 0.0001$ compared with PA 400 μ M at the same time point, ‡ $p < 0.05$ compared with 4 hrs of PA exposure.

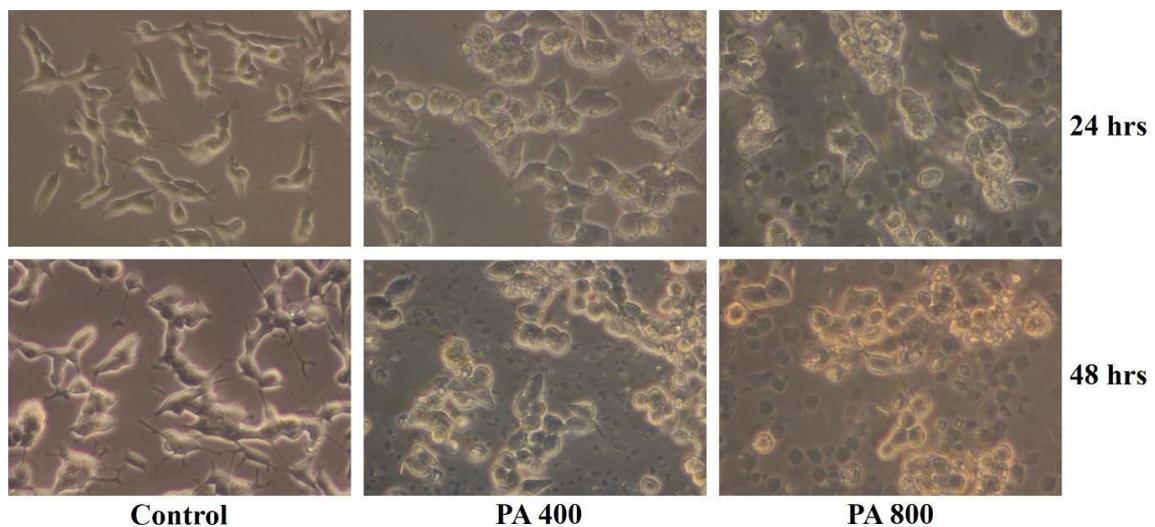


Figure 3: Image of SH-SY5Y cell line after exposure to 400 and 800 μ M of PA for 24 and 48 hrs by light microscope with a magnification of 400X.

The Effects of PA on the Expression of Alzheimer's-Related Proteins of SH-SY5Y Cells

The results demonstrated that SH-SY5Y cells exposed to 400 and 800 μ M of PA for 48 hrs significantly increased A β /APP ratio and Tau proteins when compared to the control group (Figure 4A, B). Moreover, exposure of 800 μ M of PA for 48 hrs also showed significantly increased expression of p-Tau proteins when compared to the control group (Figure 4C). However, PA at the concentration of 400 μ M did not affect p-Tau proteins when compared with control (Figure 4C). The representative band was displayed in Figure 4D.

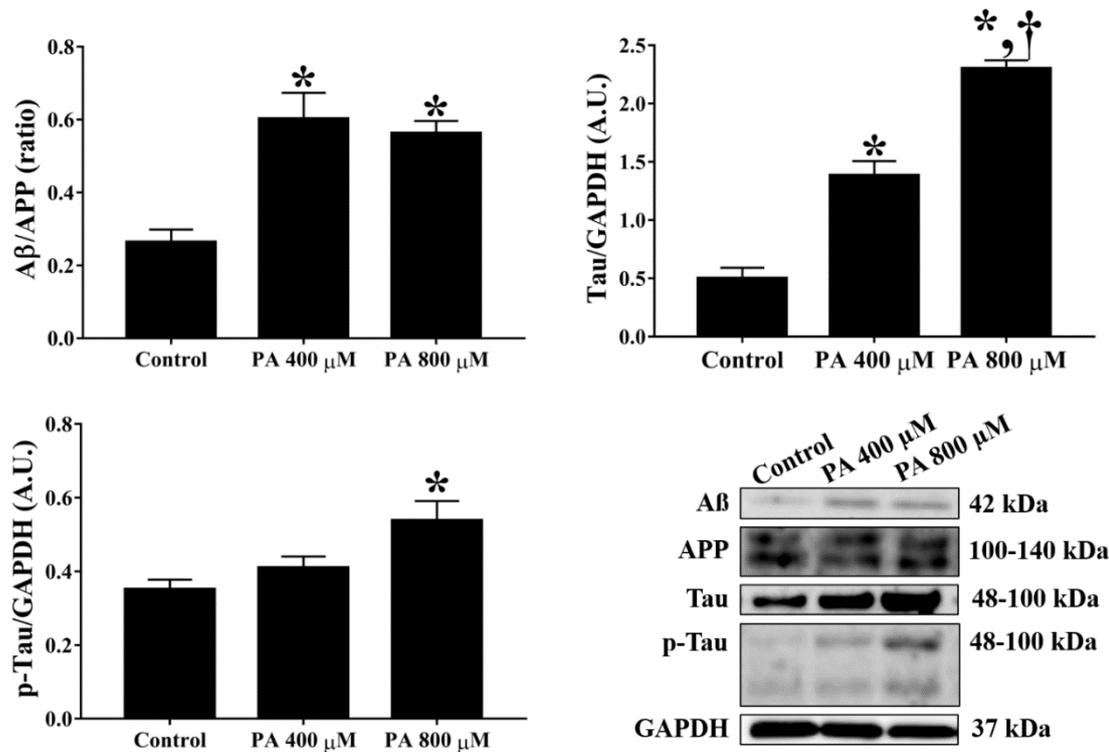


Figure 4: The effects of PA exposure at the concentration of 400 and 800 μM for 48 hrs on the expression of Alzheimer's-related proteins in SH-SY5Y cells. Western blot analysis and relative bar graph quantification of proteins (A) Aβ/APP, (B) Tau and (C) p-Tau that normalize with GAPDH and (D) representative bands. N = 4 per group in each time point. The values are presented as mean ± SEM. * $p < 0.05$ compared with control group.

Discussion

Consumption of HFD not only induces obese condition, it is also one of the major risk factors in the development of cognitive decline, dementia, and AD (Eskelinen *et al.*, 2008). Furthermore, PA, a saturated fatty acid, has been shown to be one of the major risk factors for the development of AD (Klein *et al.*, 2005; Marwarha *et al.*, 2017). Regarding to the results of this study, we found that PA affected to cell viability of SH-SY5Y cells since both 400 and 800 μM of PA significantly reduced the percentage of cell viability by dose-dependent manner. Moreover, exposure of PA for 4 and 24 hrs at each dose of PA also presented the reduction of cell viability by time-dependent manner significantly. However, when SH-SY5Y cells exposed to PA for 48 hrs, the percentage of cell viability was not different from 24 hrs of exposure. To illustrate this point, if we further extend the incubation period of SH-SY5Y cells with PA, the cell viability may become the worse and show the reduction in comparison to 24 hrs of PA exposure. Furthermore, previous study showed that HFD consumption increased Aβ and Tau proteins in the brain of 3xTg-AD mice (Julien *et al.*, 2010). However, previous studies of PA affected to the expression of Aβ together with Tau proteins in SH-SY5Y neuronal cells have not been investigated. The present study reported that PA caused the upregulation of the Alzheimer's related proteins production such as Aβ peptides, p-Tau and Tau proteins in SHSY5Y cells. Consequently, our study provided that high concentration of PA plays a vital role in the upregulation of neuronal toxicity which is related to Alzheimer's pathology and neuronal cell death.

Conclusion

The results illustrated that PA was found to be a neurotoxic substance to neuronal cells as shown by upregulated levels of proteins associated with the development of Alzheimer's pathology, including A β peptides, p-Tau and Tau proteins. Moreover, these Alzheimer's related proteins were found related to the increasing of neuronal cell death. Our study suggests that long-term of HFD consumption can cause not only obesity, but also damage to neuronal cells, resulting in brain inflammation, brain cell death and further the development of Alzheimer's disease.

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