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ROLE OF ORIENTIN AGAINST BETA-AMYLOID INDUCED CELL DEATH IN SH-SY5Y HUMAN NEURONAL CELLS

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Abstract

Alzheimer's disease (AD) is the most common form of dementia. Accumulation of beta-amyloid (Aβ) plaque has led to anti-inflammatory response, tau hyperphosphorylation and eventually neuronal apoptosis. Current treatment options only reduce the symptoms of AD but not curing the root cause of the problem. This brings us to a search for potential drugs that could tackle the cause of AD. Past studies have shown that orientin is neuroprotective due to its antiapoptotic, anti-inflammatory and antioxidative properties. Hence, this study aims to investigate the mechanism of action of orientin against Aβ-induced cell death in SH-SY5Y human neuronal cells. The SH-SY5Y cells were pre-treated with maximum non-toxic dose (MNTD) and half MNTD (½ MNTD) of orientin (20 µM and 10 µM, respectively) for 4 hours and further exposed to the inhibitory concentration of 50% (IC_{50}) of Aβ (53 µM) for 24 hours. After pre-treating the Aβ-challenged cells with orientin, the cell apoptosis, caspase 3/7, 8 and 9 assays as well as the expression of mitogen-activated protein kinase (MAPK) proteins were determined. MNTD, ½ MNTD and IC_{50} were pre-determined using 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Cell apoptosis was evaluated by Hoechst staining and changes in caspases were determined using luminescent assay kits. Finally, protein detection was carried out using western blot. The percentage of apoptotic cells exposed to Aβ was reduced when pre-treated with orientin either at MNTD or ½ MNTD. Findings from caspase activities revealed a reduction in caspase 3/7 activities when pre-treated with orientin at MNTD. Analysis of the MAPK pathway revealed that orientin at ½ MNTD could downregulate the expression of p38 and JNK proteins. This study demonstrated that orientin could inhibit Aβ-induced cell death in SH-SY5Y cells through the MAPK signalling pathway and caspase cascade.

INTRODUCTION

Neurodegenerative disease is one of the major threats faced by the elderly population globally. Alzheimer's disease (AD) is the most common form of dementia and many factors contribute to AD, such as age, genetics and various comorbidities [1, 2]. It is estimated that 5.8 million of America's population of all ages are battling with AD in 2019 and 5.6 million Americans living with AD are 65 years and older [3]. Among the 5.6 million AD Americans, 3.5 million are females and 2.1 million are males [4]. Females are at a higher risk of developing AD due to reduced estrogen levels after menopause [5]. Accumulation of β-amyloid (Aβ) has been the mainstream hypothesis underlying the pathogenesis of AD, on top of other hypotheses such as the tau hypothesis, oxidative stress hypothesis and inflammatory hypothesis [3]. Accumulation of Aβ plaque has led to anti-inflammatory responses, tau hyperphosphorylation and

eventually neuronal apoptosis [6, 7]. Studies conducted in the past demonstrated an increase in expression of p38 and JNK proteins, which are involved in the mitogen-activated protein kinase (MAPK) pathway in AD [8]. A number of caspases were also found to be upregulated in AD patients [9]; specifically, upregulation of caspase 3/7, caspase 8 and caspase 9 activities was noted in AD [9].

Currently, treatments used for AD only reduce the symptoms of AD but does not cure the disease. For examples, cholinesterase inhibitors prevent the breakdown of acetylcholinesterase and N-methyl-D-aspartate (NMDA) antagonists and further prevent glutamate excitotoxicity in the brain [10]. This brings to a continued search for potential drugs that could tackle the cause of AD. In order to combat the disease, prevention of neuronal apoptosis is crucial. Extensive research on flavonoids were done due to their tremendous healing properties. Flavonoids are natural compounds found in fruits and vegetables [11]. One of the famous flavonoids, named as orientin, is a *C*-glycosyl flavonoid that mainly isolated from a plant called *Ocimum sanctum* Linn (*O. sanctum*) or commonly known as the 'Indian Holy Basil' [12]. *O. sanctum* is well known in alternative medicines such as Ayurveda [13]. Its leaves are commonly used in the treatment of diseases such as common cold, skin lesions (for example ringworm and eczema), high blood pressure and indigestion [14]. Past studies on orientin had shown that it possessed anti-apoptotic, anti-inflammatory, antioxidative and neuroprotective properties [13]. Furthermore, orientin could reduce the cognitive deficit in mice affected by AD. After being treated with orientin, the oxidative stress biomarkers such as reactive oxygen species (ROS), 3-nitrotyrosine (3-NT) and 4-hydroxynonenal (4-HNE) in the AD mice were reduced significantly [15]. In addition, mitochondrial dysfunction caused by Aβ was found reduced by the orientin treatment. These studies had shown that orientin might be a potential drug to treat AD [15]. However, there are no previous studies which demonstrated the neuroprotective effects of orientin against Aβ-induced cell death. Therefore, current study investigated the effects of orientin against cell death by using Aβ-induced SH-SY5Y cells as the AD *in vitro* model. This study aims to determine the mechanism of action of orientin against Aβ-induced cell death in SH-SY5Y human neuronal cells.

MATERIALS AND METHODS

Preparation of Orientin

The orientin used was purchased from Sigma-Aldrich, USA and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) to produce a stock solution of 44.6 mM.

Preparation of $A\beta_{1-42}$

Aβ was manufactured by GenScript, USA. Stock solution of 2.77 mM was prepared by dissolving Aβ in DMSO. $A\beta_{1-42}$ was used throughout the whole study.

Cell Culture

The SH-SY5Y cell line was acquired from American Tissue Culture Collection (ATCC). The cells were cultured in 25 cm^2 tissue culture flask (Corning, USA) containing Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS; Gibco, South America), 1 % penicillin and streptomycin (GIBCO, South America) and 0.1 % fungizone (Gibco, South America). These cells were incubated at 37 °C with 5 % carbon dioxide in a humidified incubator (RS Biotech, UK).

Treatments

Treatments were carried out to determine the neuroprotective effects of orientin on SH-SY5Y cells. The six treatment groups were (1) Control (cells + DMSO), (2) cells + 53 μM Aβ, (3) cells + 10 μM orientin, (4) cells + 20 μM orientin, (5) cells + 10 μM orientin + 53 μM Aβ, and (6) cells + 20 μM orientin + 53 μM β-amyloid. In this study, the cells were pre-treated with maximum non-toxic dose (MNTD) and half MNTD of orientin (20 μM and 10 μM, respectively) as determined in the previous study conducted by Law *et al.* [16]. After 4 hours of incubation, the cells were exposed to 53 μM of Aβ, which is the inhibitory concentration of 50 % (IC_{50}) as determined by 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (data not shown). After 24 hours, the cells were subjected to cell apoptosis assay, caspases activity and western blot analyses.

Cell Apoptosis Assay

A flat bottom 6-well plate (Corning, USA) was used to seed $1x10^6$ cells/mL of SH-SY5Y cells in each well. When 70 % of cell confluency was achieved, the cells were subjected to various treatments. After the treatments, media in the 6-well plate were removed and the cells in each well were washed with phosphate-buffered saline (PBS) thrice. Next, 70 % of methanol at 4 °C was added into the wells and incubated in -20 °C for 30 minutes. After that, the methanol was removed, and the cells were air dried. The cells were then washed with PBS thrice and 1 μL of Hoechst 33258 stain (1 mg/mL; Sigma-Aldrich, USA) was added to each well. The cells were incubated for 10 minutes in the dark at room temperature. The staining solution was aspirated from each well and the cells were washed with PBS once. Finally, the

cells were viewed under fluorescence microscope (Nikon, Japan) with 4′,6-diamidino-2-phenylindole (DAPI) filter. Micrographs under 200X magnification were captured. In this study, the number of apoptotic cells that are characterized by condensed chromatin and fragmented nuclei was quantified. These two characteristics were observed as increased blue colour intensity under the fluorescence microscope. The total cell number in a field (ranging from 20-2000 cells) was also counted.

Measurement of Caspases Activities

In this study, 1 x 10^6 cells/mL of SH-SY5Y cells were seeded into white flat bottom 96-well plate (Nunc, Denmark). Once the cells reached 70 % confluency, they were subjected to the treatments. This was followed by the measurement of activities of caspase 3/7, 8 and 9 using Promega Caspase-Glo® 3/7, Caspase-Glo® 8 and Caspase-Glo® 9 assay kits (Promega, USA), respectively. Manufacturer's instruction was adopted as guidelines for measurement. A luminometer Spectramax 3 (Biocompare, USA) was used to read the luminescence.

Western Blot Analysis

A total of 1 x 10^6 cells/mL of SH-SY5Y cells were seeded in each well of a clear flat bottom 6-well plate (Nunc, Denmark). When the cells reached 70 % confluency, they were subjected to treatments. After the incubation period, medium from each well was collected into respective 3 mL centrifuge tube and the tubes were spun for 5 minutes at 1500 rpm. After the supernatant was removed, the remaining pellet of dead cells were kept for protein extraction. Then, 209 µL of lysis buffer was added in each well and the cells were scrapped off using a cell scrapper (TPP, Switzerland). The cells that were scrapped off and the pellet obtained previously were mixed in a 1.5 mL microcentrifuge tube. The respective microcentrifuge tubes were centrifuged at 1500 rpm for 5 minutes and the supernatant which contained the proteins were collected and kept in -80 °C freezer prior to further analysis.

The protein samples (0.75 mg/mL) were separated by 10 % sodium dodecyl sulphate (SDS) polyacrylamide gel for an hour at 80 V and then at 100 V for the second hour using a Powerpac Basic Machine (Bio-Rad, USA). After the running process, proteins in the gel were transferred to a 0.45 µM polyvinylidene difluoride (PVDF) membrane (Immobilon, USA). This was followed by 2 hours of incubation of membrane in a blocking solution of 4 % bovine serum albumin (BSA) prepared in Tris-buffered saline in Tween 20 (TBST). After the blocking step, the membranes were incubated for 12 hours with primary antibodies, which were SAPK/JNK, p38 MAPK and β-actin (Cell Signaling Technology, UK). The primary antibodies were used at a ratio of 1:1000 in 4 % BSA-TBST.

After 12 hours incubation period, the membranes were washed 3 times with TBST for 10 minutes each time. Then, these membranes were incubated for an hour with the secondary anti-rabbit antibody conjugated with horseradish peroxidase (HRP) (Fine Test, China) at a ratio of 1:5000 in 4 % BSA-TBST. The membranes were then washed thrice for 10 minutes each time with TBS. These membranes were visualized under ChemiDoc XRS+ imaging system (Bio-Rad, USA) with the addition of chemiluminescent substrate (SuperSignal West Femto) (Thermo Scientific, USA). The different intensity of protein bands obtained were analyzed through quantitative densitometry of proteins. The densitometry data of the protein was then further analysed and normalised to the control group and expressed as relative protein expression.

Statistical Analysis

The data shown in this study were expressed as means ± S.D. The data obtained was analysed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test using SPSS version 16.0. The differences between the treatments were compared, in which $p < 0.05$ was considered as statistically significant.

RESULTS AND DISCUSSION

Cell Apoptosis Assay

As shown in Figures 1 and 2, an increase in percentage of apoptotic cells (17.86 %) was observed when SH-SY5Y cells were exposed to Aβ, compared to the control group, which was 9.6 %. Furthermore, the results have demonstrated that the percentage of Aβ-induced apoptosis could be reduced when SH-SY5Y cells were pre-treated with orientin either at ½MNTD or MNTD. Orientin at both ½MNTD and MNTD was able to decrease the percentage of apoptotic cells from 17.86 % to 10.87 % and 15.59 %, respectively.

According to the current study, the percentage of apoptotic cells was higher in the Aβ group when compared to the control cells. Similar results were reported previously, whereby $A\beta_{1-42}$ induced neuronal apoptosis by targeting mitochondria in mouse cerebral cortical neurons [17]. The results reported by Jang et al. showed disappearance of red-orange fluorescence after tetramethylrhodamine, ethyl ester (TMRE) stain was used on PC12 cells that were exposed to Aβ [18]. These results indicated that Aβ exposure could cause a reduction in cell viability [19]. These findings are in accordance with the current study, which reported the ability of β-amyloid in triggering the apoptosis.

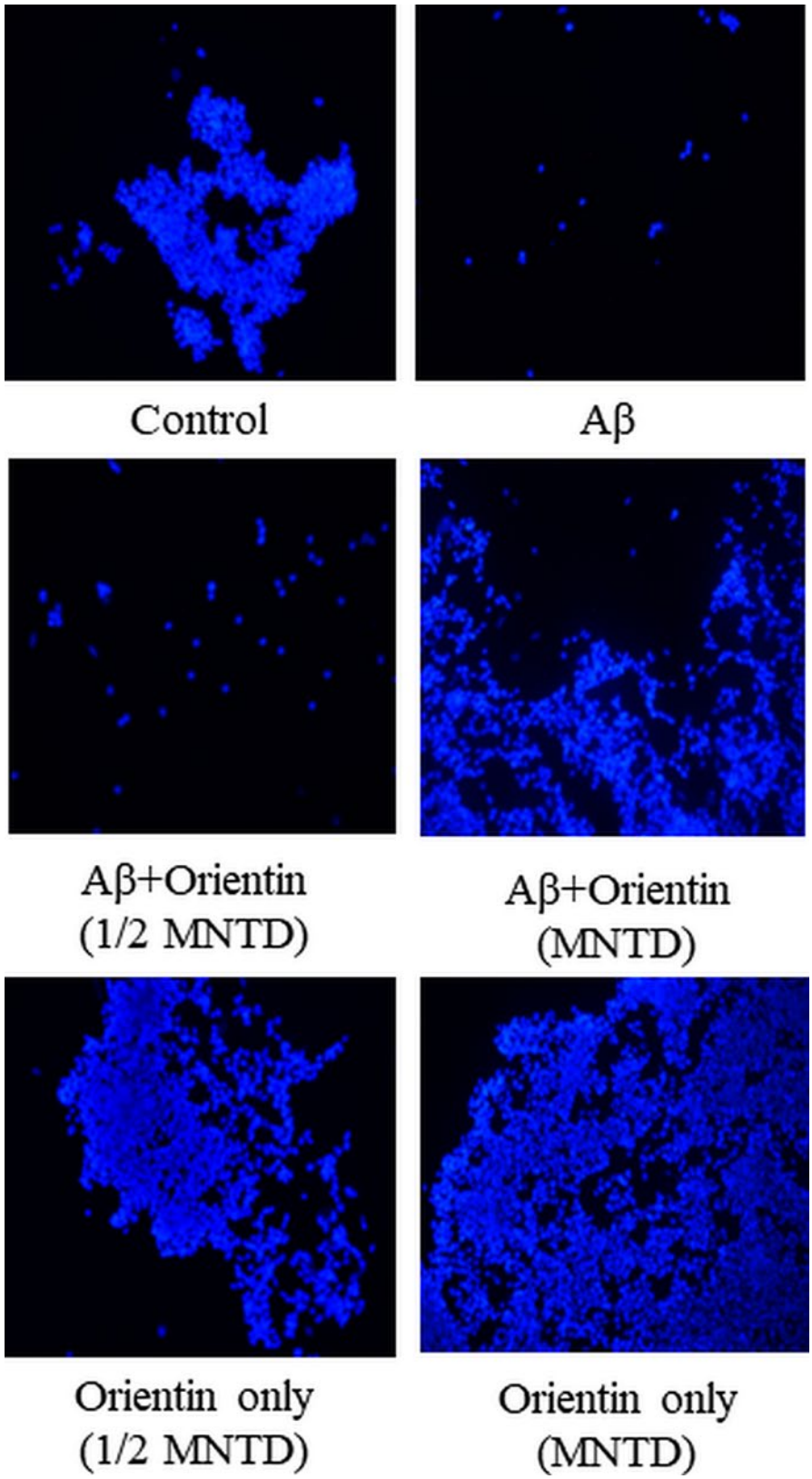

Figure 1. The effects of orientin on SH-SY5Y cell apoptosis. The cells were stained with Hoechst stain. Magnification: 200x

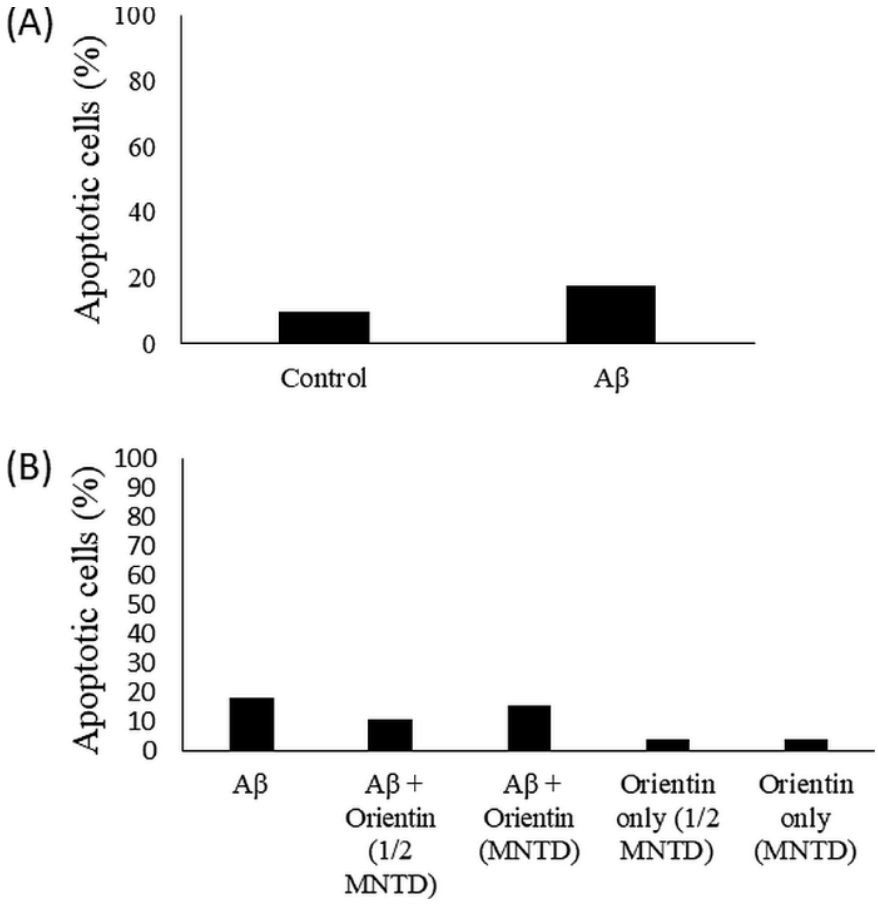

Figure 2. The effects of orientin on percentage of apoptotic cells. (A) SH-SY5Y cells were treated with beta-amyloid (Aβ) alone. (B) Aβ-induced SH-SY5Y cells were treated with orientin at MNTD and ½ MNTD

The main objective of this study was to analyse the effects of orientin on apoptosis. Based on the current findings, the cells that were pre-treated with ½MNTD and MNTD of orientin and exposed to Aβ reported a reduction in percentage of apoptotic cells, when compared to Aβ alone. The present study proves that orientin possessed anti-apoptotic effect, which is in line with the previous study on flavonoids, such as luteolin and myricetin, that showed to decrease the percentage of apoptosis in mouse cortical neurons [20]. Another study also reported that flavonoids like epigallocatechin gallate and quercetin decreased the rate of hydrogen peroxide (H_2O_2)-induced cell apoptosis in human umbilical vein endothelial cells (HUVEC). HUVEC that were pre-treated with epigallocatechin gallate and quercetin had demonstrated a reduction in nuclear condensation and DNA fragmentation [21]. The anti-apoptotic effects of orientin could be due to its anti-inflammatory property as it can suppress the production of proinflammatory cytokines [22]. Orientin was also found to prevent cell death through inhibition of several apoptotic pathways, for example MAPK pathway and caspase cascade [23].

Measurement of Caspases Activities

Figure 3 shows the levels of caspase 3/7, 8 and 9 in SH-SY5Y, after subjected to the various treatment groups as mentioned before. A significant increase in the caspase 3/7activities was noted upon exposure to Aβ (Figure 3A). The ½MNTD orientin only and MNTD orientin only groups presented with significantly lower level of caspase 3/7 activities when compared to the Aβ group. As for the treatment groups which were exposed to Aβ, caspase 3/7 activities were reduced when cells were pre-treated with MNTD orientin. However, pre-treatment with ½MNTD orientin did not show a reduction in the caspase 3/7 activities. This might be due to the fact that low dose of orientin was insufficient in rescuing the cell damage caused by Aβ.

Contrary, ½MNTD orientin only and MNTD orientin only presented with higher levels of caspase 8 activity compared to the Aβ group (Figure 3B). Pre-treatment with ½MNTD of orientin to the Aβ-induced cells did not lead to a reduction in the caspase 8 activity. Nevertheless, pre-treatment with MNTD orientin for the cells that were exposed to Aβ led to a slight decrease in the caspase 8 activity ($p > 0.05$).

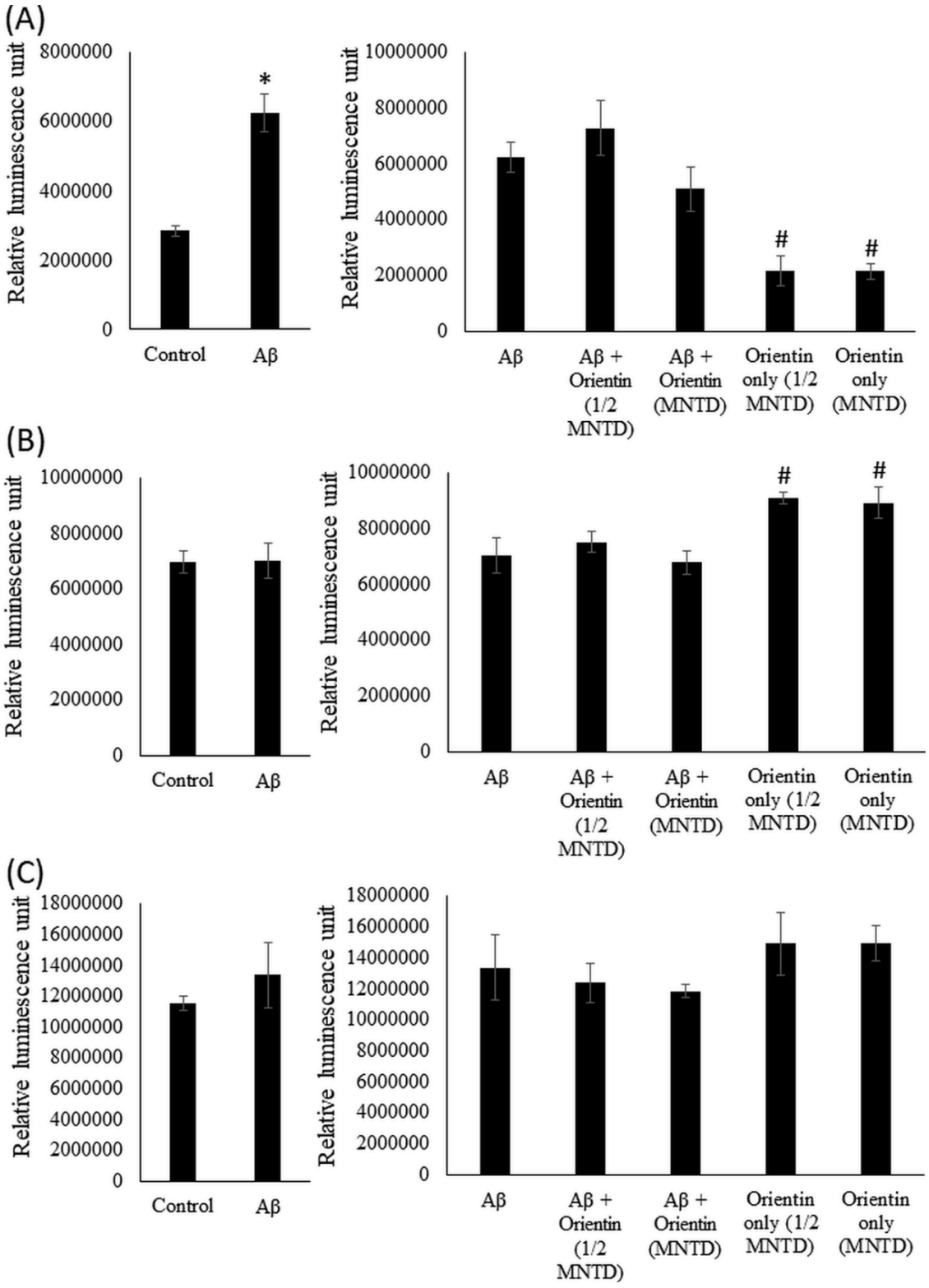

Figure 3. The effects of orientin on caspases activities. (A) Caspases 3/7, (B) caspase 8, and (C) caspase 9. The data shown are means ± S.D. of three replicates. '*' denotes the treatment was significantly different from the control cells while '#" denotes the treatment was significantly different from the beta-amyloid (Aβ), analysed using one-way analysis of variance followed by Tukey's multiple comparison test at $p<0.05$

In Figure 3C, among the three treatment groups which were exposed to Aβ, the cells which were pre-treated with ½MNTD orientin (10 μM) and MNTD orientin (20 μM) demonstrated a slight reduction in caspase 9 activity compared to the group of Aβ only.

In this study, cells that were exposed to Aβ presented with a significant increase in caspase 3/7 activities. The results indicated that Aβ could stimulate caspase 3/7 activation, which led to neuronal apoptosis through the execution pathway. This statement is supported by the evidence from research done on primary cultures of rat neurons, which reported Aβ induced activation of caspase 3 [16]. Pre-treatment with orientin at MNTD, followed by exposure to Aβ, presented a reduction in the caspase 3/7 activities, which demonstrates that orientin could possibly possess a neuroprotective effect on SH-SY5Y cells. The neuroprotective effects of orientin were reported in a study conducted by Law et al., in which orientin inhibited the expression of caspase 3/7 and led to the reduction of intracellular ROS levels in SH-SY5Y cells [16]. Furthermore, Sharma et al reported that quercetin - a type of flavonoid, reduced the expression of cytochrome C and downregulated the activity of caspase 3 in cells from the rat hippocampus [24].

The present study also revealed that the SH-SY5Y cells, which were pre-treated with ½MNTD orientin followed by 53 μM Aβ showed an increase in caspase 3/7 activities. There are studies which showed that flavonoid might cause an increase or decrease in caspases activities depending on the different molecular interactions under specific concentrations and conditions. A study on colorectal carcinoma cells (HT29 cells) showed that orientin activates caspase 3, which further suggested that orientin could induce apoptosis through stimulation of caspase cascade [25]. Nevertheless, another possible explanation for the increase in caspase 3/7 might be due to non-apoptotic effects of caspases. Previous study had reported that caspase 3 plays a role in the regulation of synaptic plasticity, whereby proteins that contribute to neuronal plasticity are substrates of caspase 3 [26]. Findings by D'Amelio et al. have also shown that upregulation of caspase 3 is associated with improvement in memory and learning in adult rats [27]. Therefore, the upregulation of caspase 3 as observed in the present study is not necessarily correlated with apoptosis.

The present study also showed that the orientin treatment groups, which were then exposed to Aβ, showed downregulation of activities of caspase 8 and caspase 9, compared to the Aβ alone group. The decrease in procaspases 8 and 9 levels may be explained by the late activation of caspase 8 and caspase 9 [28]. Although apoptosis is usually associated with upregulation of caspase expression, it could take into consideration that initially caspases are synthesized as zymogens and only activated due to specific stimulus [29]. The downregulation of caspase 8 by orientin in the Aβ-induced cells might be due to phosphorylation of caspase 8 by p38 MAPK. Alvarado-Kristensson et al. reported that phosphorylation of caspase 8 by p38 MAPK at Ser364 inhibited the active caspase and protected neutrophils from Fas-induced death [30]. As the present study showed an increase in p38 expression in the orientin-treated groups, this could possibly explain that the upregulation of p38 expression might have inhibited the activation of caspase 8.

Caspase 8 activity in the Aβ-induced cells was not inhibited by ½MNTD orientin, as a slight upregulation in caspase 8 activity was observed. Research in the past had also shown that some flavonoids could induce caspase 8 activity under certain circumstances. For instance, a study on the effects of C-glucoside on human breast adenocarcinoma (MCF-7) reported an increase in the mRNA expression of caspase 8, suggesting that extrinsic pathway was contributed to the C-glucoside-induced apoptosis [31]. On the other hand, there was a slight reduction in caspase 8 activity for the cells pre-treated with MNTD orientin, followed by Aβ exposure. The results showed that a higher concentration of orientin managed to provide better protective effects against the Aβ-induced apoptosis on SH-SY5Y cells. Similarly, a previous study revealed that flavonoid like orientin could significantly reduce caspase 8 activities in H_2O_2-induced cell death of SH-SY5Y cells [16].

The decrease of caspase 9 activity in the orientin treatment groups, which were then exposed to Aβ could be related to the earlier activation of caspase 3, prior to caspase 9. Although caspase 9 is known as an upstream mediator for caspase 3 in the extrinsic apoptotic pathway, the findings obtained from the previous study suggested otherwise [32]. Another possible explanation to the downregulation of caspase 8 and caspase 9 activities is that caspases are substrates of calpains. Cleavage by calpains produces inactive fragments of caspase [33]. Therefore, cleavage of caspases by calpain causes the production of inactive fragments of caspase, which led to the decrease in caspases activities as observed in the present study. A slight decrease in caspase 9 activity was shown in cells pre-treated with ½MNTD orientin + 53 μM Aβ and MNTD orientin + 53 μM Aβ. Similarly, a previous study on rat hippocampal neurons presented with a reduction in transcription levels of caspase 3, caspase 8, caspase 9, Cytochrome C and Bax due to treatment with Gingko flavonoids [34]. In addition, a flavonoid called fisetin was reported to downregulate the expression of cleaved caspase 3 and caspase 9, Cytochrome C and Bax in $A\beta_{1-42}$ mouse model of AD.

Expression of MAPK Proteins via Western Blot Analysis

As illustrated in Figures 4 and 5A, the treatment group which was pre-treated with ½MNTD orientin followed by Aβ showed a reduction in p38 expression, while the treatment group which was pre-treated with MNTD orientin followed by Aβ showed an increase in p38 expression when compared to the cells exposed to Aβ only. The present study revealed that Aβ increased the expression of SAPK/JNK (46)

compared to the untreated cells (Figure 5B). For the SH-SY5Y cells that exposed to Aβ, the group pre-treated with ½MNTD orientin led to a decrease in the expression of SAPK/JNK (46) while the group pre-treated with MNTD orientin showed a slight downregulation in the expression of SAPK/JNK (46) compared to the group of Aβ only. The present study also revealed that by exposing the cells to 53 µM of Aβ, pre-treatment of orientin at MNTD and ½MNTD reduced the expression of SAPK/JNK (54) as compared to the cells exposed to Aβ only.

In this study, the SH-SY5Ycells which were exposed to Aβ had an increased p38 expression. This shows that Aβ stimulated the expression of p38 during apoptosis. This finding is supported by a study involving mouse model that was exposed to Aβ deposits and recorded an increase in p38 phosphorylation [35]. The treatment groups, which were exposed to 53 µM of Aβ and pre-treated with ½MNTD orientin presented with a downregulation in the expression of p38. Hence, this result revealed that orientin protected the SH-SY5Y cells from apoptosis through the p38 pathway. In fact, previous study reported that flavonoids like naringenin produced anti-inflammatory effects by inhibiting p38 MAPK phosphorylation in lipopolysaccharides (LPS)-stimulated primary mixed glial cells [36]. However, the cells that were pre-treated with MNTD orientin + 53 µM Aβ showed upregulation in the expression of p38. This could possibly be due to the fact that a higher concentration of orientin might have triggered the expression of p38 proteins. This result could be associated with a study conducted previously, which demonstrated an increase in phosphorylation of p38 MAPK in human glioblastoma cells (T98G and U87MG cells) after treatment with flavonoids (apigenin, epigallocatechin, epigallocatechin-3-gallate and genistein). Previous study proved that higher concentration of flavonoid might be cytotoxic to cells and subsequently led to an increase in expression of p38, which eventually caused apoptosis [37].

In this study, Aβ induction in SH-SY5Y demonstrated an upregulation in the expression of SAPK/JNK (46) and SAPK/JNK (54) as compared to the control cells. Previous study revealed the JNK activation in abnormal neurites that contained phosphorylated tau surrounding deposits of Aβ [38]. Therefore, findings from the previous study are in accordance with the current study, which demonstrated an upregulation of SAPK/JNK after exposure to Aβ.

As for the groups pre-treated with MNTD and ½MNTD orientin followed by exposure to Aβ, decrease in SAPK/JNK (46) and SAPK/JNK (54) expression was observed comparing to the group exposed to Aβ alone. This could be due to the neuroprotective effect of orientin in reducing the expression of SAPK/JNK of the extrinsic and intrinsic signaling apoptotic pathways. Neuroprotective effects of flavonoid were also demonstrated in past study, where rutin was shown to suppress the expression of JNK and p38 proteins expression levels in isoflurane-induced neuroapoptosis of hippocampi of neonatal rats [39]. Another study presented that flavonoid-rich tartary buckwheat sprout (TBS) extract inhibited the expression of phosphorylated-JNK and phosphorylated-ERK in LPS-induced RAW 264.7 macrophages [40].

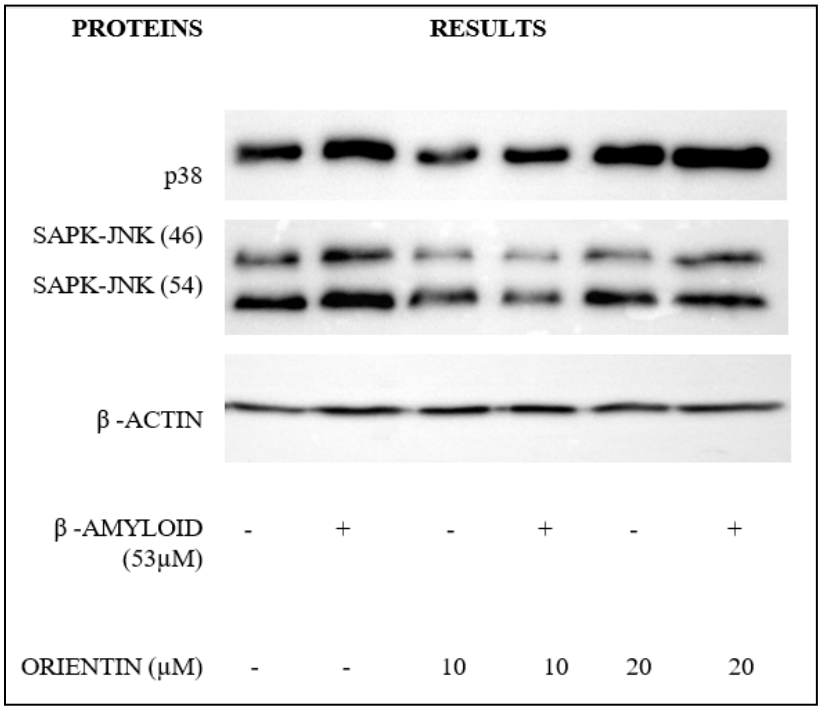

Figure 4. Western blot analysis results for different MAPK proteins; p38, SAPK-JNK (46), SAPK-JNK (54) and β-actin (housekeeping gene) after SH-SY5Y cells were pre-treated with orientin for 4 hours, followed by β-amyloid challenge for 24 hours

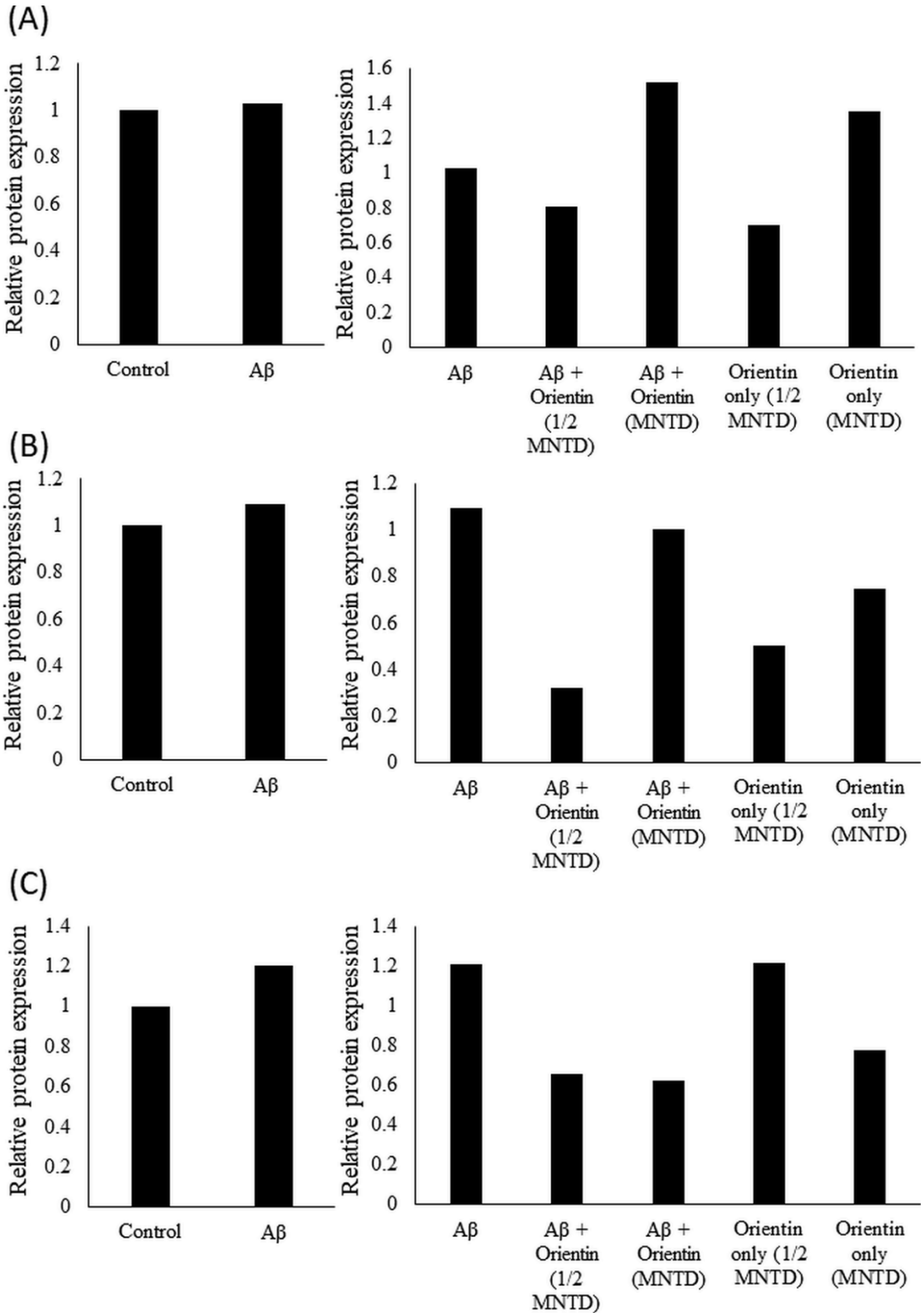

Figure 5. The effects of orientin on proteins expression in SH-SY5Y cells. (A) p38, (B) SAPK/JNK (46), and (C) SAPK/JNK (54)

CONCLUSION

The mechanism of orientin determined in this study is via the caspase enzyme system, especially caspase 3/7 and the inhibition of MAPK pathway, which is mainly mediated through p38 and JNK proteins. However, the underlying mechanism of orientin remains unknown, and this warrant further investigations. For instance, the decrease in p38 and JNK expression could be due to the inhibition of Ras activation or the kinase cascade (MEK), which prevents the phosphorylation of the MAPKs (p38 and JNK). Further analysis of the expression of RAS and MEK proteins would provide a better understanding of the effects of orientin on the MAPK pathway.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this manuscript.

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