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The recovery and protective effects of asiatic acid on differentiated human neuroblastoma SH-SY5Y cells cytotoxic-induced by cholesterol

Kanchanat Ternchoocheep[1*], Damrassamon Surangkul[1], Sukhgij Ysothonsreekul[2]

[1]*Department of Biochemistry, Faculty of Medical Science, Naresaun University, Muang, Phitsanulok 65000, Thailand*

[2]*Department of Scientific Research, PTT Global Chemical Public Company Limited, Noenphra, Mueang Rayong, Rayong 21150, Thailand*

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ABSTRACT

Objective: To investigate the effect of asiatic acid (AA) on the differentiated human neuroblastoma SH-SY5Y cells cytotoxic-induced by cholesterol.
Methods: Human neuroblastoma SH-SY5Y cells were either exposed to different concentrations of AA or treated with different doses of cholesterol to reveal their responding viability by MTT assay. The selective 1 μmol/L concentration of AA was then used to test for either the protective or the recovery effects on the cells treated with 250 μmol/L concentration of cholesterol.
Results: AA has a propensity to directly increase the viability of differentiated human neuroblastoma SH-SY5Y cells. Cholesterol has significant cytotoxic effect on those cells in a concentration-dependent manner. AA has the ability to slightly recover the viability of the differentiated culture cytotoxic-induced by cholesterol but could not protect those cells from cytotoxic-induced by cholesterol.
Conclusions: High concentrations of cholesterol were observed to be harmful to the neurons and AA had a slight effect of reducing neuronal death caused by cholesterol.

1. Introduction

Alzheimer's disease, a neurodegenerative disease, is a major problem in geriatric medicine. The putative pathologies of the disease include extracellular aggregation of amyloid-β (Aβ) in the form of senile plaques and intracellular inclusion of hyperphosphorylated tau in the form of neurofibrillary tangles (NFTs). Recently, evidence of the association of cholesterol to Alzheimer's disease pathogenesis has been published. Cholesterol is one of the main components of neuronal membrane. In the neuronal membrane, cholesterol is concentrated in microdomains termed "lipid rafts" [1]. Amyloid precursor protein (APP), β-secretase, and γ-secretase co-exist in the lipid rafts, so the amyloidogenic process mostly occurs in this site [2]. Cholesterol specifically binds to the β-C-terminal fragment of the APP and enables it to localize in the lipid rafts [3]. With increased membrane cholesterol, APP and β-secretase are in closer proximity inside the neurons [4]. Additionally, enriched cholesterol in the lipid rafts facilitates the cleavage of APP into Aβ peptide [5]. Some studies report that increased levels of extracellular free cholesterol are toxic to neurons in a dose-dependent manner [6] and reducing membrane cholesterol content can lessen the cytotoxicity induced by monomeric Aβ [7].

Centella asiatica (L.) Urban (*C. asiatica*) (Syn. *Centella coriacea* Nannfd., *Hydrocotyle asiatica* L., *Hydrocotyle lunata* Lam., and *Trisanthus cochinchinensis* Lour.) is a tropical medicinal plant from the Apiaceae family which is native to South and Southeast Asian countries including India, Sri Lanka, China, Indonesia, and Malaysia, and is also found in South Africa and Madagascar [8]. In Ayurvedic medicine, *C. asiatica* has been utilized for restoring memory and longevity and has been used, in traditional Chinese medicine as well, to combat physical and mental exhaustion [9]. The important chemical

*Corresponding author: Dr. Kanchanat Ternchoocheep, Department of Biochemistry, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand.
Tel: +66 88 8856374
Fax: +66 55 964770
E-mail: kternchoocheep@gmail.com

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components found in this plant are triterpene saponosides such as asiatic acid (AA), madecassic acid (6-hydroxy-asiatic acid), asiaticoside, madecassoside, and madasiatic acid [8]. Long-term *C. asiatica* treatment has been shown to significantly improve memory performance (increased memory retention) in memory-impaired mice induced by D-galactose [10]. *C. asiatica*-fed mice display a significant diminution in the basal level of reactive oxygen species in cytosol and mitochondria [11]. *C. asiatica* extract exhibits not only a reduction of Aβ levels and fibrillary amyloid load in the hippocampus but also a significant reactive oxygen species scavenging activity in treated mice [12]. In ischemic rats, the antioxidant activity of *C. asiatica* is putatively attributed to its bioactive triterpenes such as AA [13].

Some studies have shown that AA improves memory and enhances learning abilities in treated Spraque-Dawley rats [14]. AA can break down the acetylcholinesterase (AChE) enzyme more effectively than the AChE inhibitor or the phytostigmine and galatamine in TLC bioautographic assay which then blocks the excitatory transmission at the hippocampal Schaffer collateral-CA1 synapse in a dose-dependent manner for GABAA antagonists [15]. Recent evidence has shown the cytoprotective effect of AA on neuronal cultures against glutamate-induced excitotoxicity [16], H_2O_2-based oxidative stress [17], and C_2-ceramide-induced apoptosis [18]. Thus far there have been no reports showing the effect of AA on the cytotoxic effect of cholesterol. Therefore, this current study investigated the impact of AA on human neuroblastoma SH-SY5Y cells toxic-induced by cholesterol.

2. Materials and methods

2.1. Materials

AA from *C. asiatica*, cholesterol powder, retinoic acid, and dimethyl sulfoxide (DMSO) were purchased from Sigma Life Science (Sigma Aldrich) and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) was purchased from Bio Basic Canada Inc.

2.2. Cell culture

Neuroblastoma SH-SY5Y cells (CRL-2266, ATCC) were grown in the normal way in a flask containing 10 mL DMEM/F12 medium (Gibco, CA, USA) with 10% fetal bovine serum and incubated at room temperature of 37 °C (Biosafety cabinet Class II, NUAIRE, USA). The cells were refreshed with new medium every 2–3 days and subcultured when cell the density reached between 2–4 × 10^6 viable cells/mL (100% confluency), at which point the cells were differentiated with 20 μmol/L retinoic acid for 4 days before plating into a 96-well microplate at the density of 10^4 viable cells/100 μL to each well. They were then grown for 24 h prior to further experiments.

2.3. The study of the effects of AA or cholesterol on cell viability

For the study of the effect of AA on cell viability, one set of samples of the differentiated cells were exposed to 0 nmol/L, 10 nmol/L, 100 nmol/L, 1 μmol/L, 10 μmol/L, and 100 μmol/L concentration of AA for 24 or 48 h. A separate set of the differentiated cells were treated with 0, 50, 100, 150, 200, 250, and 300 μmol/L concentration of cholesterol for either 72 or 96 h for the study of the effect of cholesterol on cell viability.

2.4. The study of recovery effect of 1 μmol/L AA

A group of differentiated cells were initially grown in a medium containing 250 μmol/L cholesterol for either 72 or 96 h before additionally exposing the cells to 1 μmol/L AA for a further 24, 48, or 72 h. An additional 5 groups of differentiated cells, which included a control group, were grown for comparison against the group just mentioned. In the control group, the differentiated cells were initially grown in the normal medium throughout the experiment. In two of the groups, designated the second and the fourth groups, the differentiated cells were raised in the normal medium initially for 72 or 96 h before exposing the cells to either 0.05% DMSO or 1 μmol/L AA respectively for an additional 24 or 48 h. In two other groups, the third and the fifth groups, the differentiated cells were raised in the medium containing either 2.5% ethanol or 250 μmol/L cholesterol since the beginning of the experiment.

2.5. The study of protective effect of 1 μmol/L AA

A group of differentiated cells were initially exposed to 1 μmol/L AA for 24, 48, or 72 h before additionally brought up in the medium containing 250 μmol/L cholesterol for further 24, 48, or 72 h. An additional 5 groups of differentiated cells, which included a control group, were grown for comparison against the group just mentioned. In the control group, the differentiated cells were grown in normal medium throughout the experiment. In two of the groups, designated the second and the fourth groups, the differentiated cells were raise in the medium containing either 0.05% DMSO or 1 μmol/L AA respectively since the beginning of the experiment. In two other groups, the third and the fifth groups, the differentiated cells were raised in the normal medium initially for 24, 48, or 72 h and then further grown in additional 2.5% ethanol or 250 μmol/L cholesterol for 24, 48, or 72 h.

2.6. MTT assay

The old culture medium was removed from each well of 96-well microplate, and 100 μL of MTT (1 mg/mL) was then added to each well and incubated further in the dark for 2 h. At the end of the MTT treatment, the medium was removed, and 100 μL DMSO was added to each well to dissolve the purple formazan crystals. The color was quantified using a synergy HT multi-mode microplate reader (BioTek, USA) at 570 nm. The viability percentage were calculated from triplicated experiments by statistical one-way ANOVA with $\alpha = 0.05$.

3. Results

3.1. Effect of AA on cell viability

The viability of the differentiated SH-SY5Y cells exposed to 0–100 μmol/L AA tended to slightly increase in a dose-dependent manner non-significantly (Figure 1). The cell viability was lower than the control's (0 nmol/L AA) when exposed to 100 μmol/L AA. Since the cells had very similar viability when exposed to 1 μmol/L and 10 μmol/L AA, we decided to use 1 μmol/L AA for our further experiments.

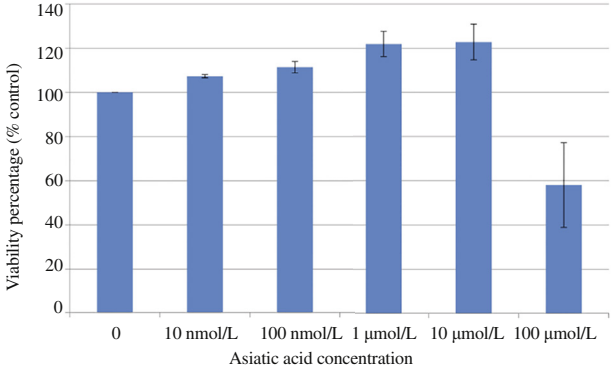

Figure 1. The viability of the differentiated SH-SY5Y exposed to 0–100 µmol/L asiatic acid.
Each bar represents mean ± SEM of 9 samples from triplicated experiments. Analysis was done by One-way ANOVA with $P > 0.05$.

3.2. Effect of cholesterol on cell viability

The viability of the differentiated SH-SY5Y cells treated with 0, 50, 10, 150, 200, 250, or 300 µmol/L concentration of cholesterol for 72 h was reduced to be lower than their control in a dose-dependent manner (Figure 2). At 200–300 µmol/L cholesterol, the viability of the treated cells was significantly different from the control. When the cells were treated with 50 and 100 µmol/L cholesterol for 96 h their viability tended to be higher than the control's non-significantly (data not shown). The cell viability following treatment with 150–300 µmol/L cholesterol for 96 h was statistically significantly lower than the control's, at the concentrations of 250 and 300 µmol/L. As the lowest concentration of 250 µmol/L provided the significant viability reduction, we decided to this concentration of cholesterol for our further experiments.

3.3. Recovery effect of AA on differentiated SH-SY5Y cells cytotoxic-induced by cholesterol

The differentiated SH-SY5Y cells were treated with 250 µmol/L cholesterol at either 72 or 96 h before exposing the cells to 1 µmol/L AA for 24 or 48 h. Those cells tended to slightly improve their viability non-significantly as compared to their corresponding cells in the fifth group which were treated only with cholesterol (Figure 3A). The viability of the cells exposed to AA for the longer period (48 h) was only slightly higher than the viability of those cells exposed for the shorter

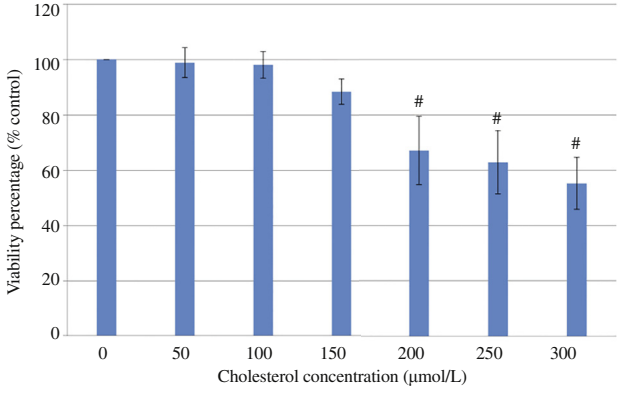

Figure 2. The viability of the differentiated SH-SY5Y cells treated with 0–300 µmol/L cholesterol for either 72 or 96 h.
Each bar represents mean ± SEM of 12 samples from triplicated experiments. #: $P < 0.05$ as compared to 0 µmol/L of 72 h cholesterol treatment.

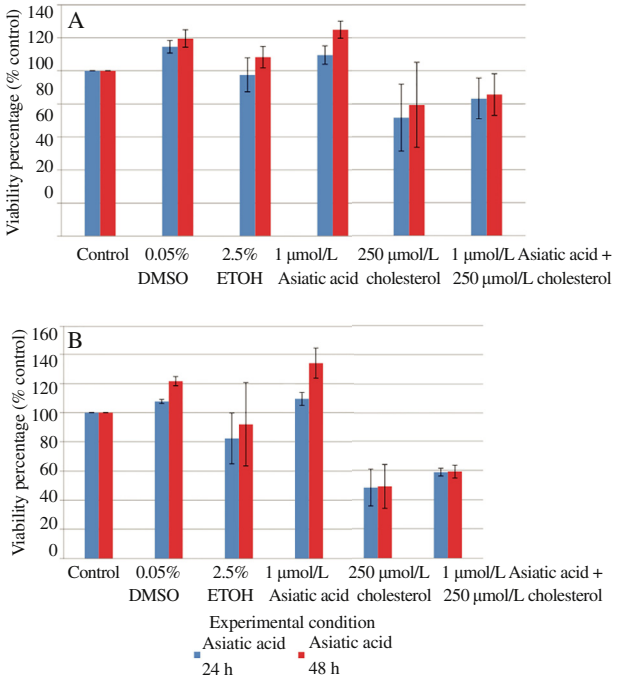

Figure 3. Average viability percentage of the differentiated SH-SY5Y cells exposed to asiatic acid after treating with cholesterol for (A) 72 h or (B) 96 h.
Each bar represents mean ± SEM of 9 samples from triplicated experiments. Analysis was done by One-way ANOVA with $P > 0.05$.

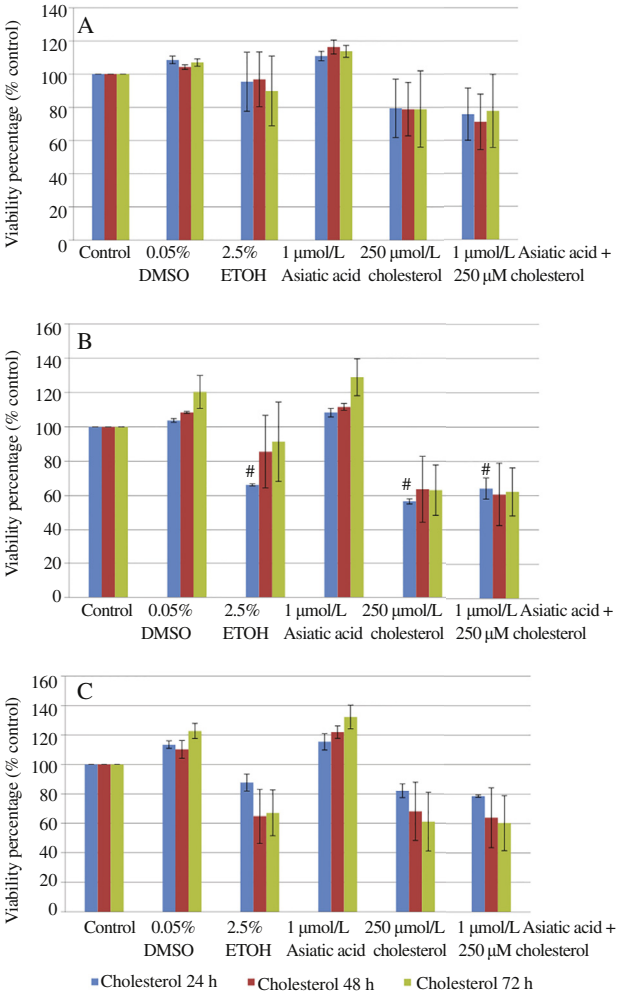

Figure 4. Average viability percentage of the differentiated SH-SY5Y cells exposed to asiatic acid for (A) 24 h, (B) 48 h or (C) 72 h before treating with cholesterol.
Each bar represents mean ± SEM of 9 samples from triplicated experiments. #: $P < 0.05$ as compared to the control of 48 h cholesterol treatment.

period (24 h) after being treated with cholesterol for 72 h (Figure 3A). The longer period of cholesterol treatment (96 h) inhibited the improvement of the cell viability instigated by the AA (Figure 3B).

3.4. Protective effect of AA on differentiated SH-SY5Y cells cytotoxic-induced by cholesterol

The differentiated SH-SY5Y cells were exposed to 1 μmol/L AA at 24, 48, or 72 h before treating the cells with 250 μmol/L cholesterol for 24, 48, or 72 h. Those cells had relatively similar results as their corresponding cells in the fifth group which had been treated with cholesterol only (Figure 4A–C). The different periods of AA exposure made no difference on the viability of those cells.

4. Discussion

A previous study [19] showed that cholesterol at the physiological dose (16 μmol/L) is not cytotoxic to androgen-dependent prostate cancer cell line PC-3 and DU145. At this concentration, cholesterol is required for the mitosis. Cholesterol at the concentration of 20–80 μmol/L lessens the proliferation of fetal rat enterocytes and IEC-6 cells [20] and at the concentration of 25–100 μmol/L weakens the viability of gastric cancer cells in a dose-dependent manner [21]. Our present study provides additional data demonstrating that extracellular cholesterol at high concentrations (50–300 μmol/L) could contribute to the cytotoxicity, which is in accordance with the previous studies cited here.

Our study demonstrated the direct effect of AA (0 nmol/L–100 μmol/L) on the human neuroblastoma SH-SY5Y cells. The viability results (Figure 1) are in accordance with a recent study in which showed that 10–100 μmol/L AA strongly reduces human glioblastoma multiform cell viability by inducing endoplasmic reticulum stress and apoptosis [22]. AA has been shown to have a protection effect against Aβ-induced neurotoxicity [23]. In primary cortical culture, pretreatment with AA (0.01–1.0 μmol/L) is neuroprotective against C_2-ceramide-induced cell death [18]. C_2-ceramide causes neuronal apoptosis. At 1 μmol/L concentration, AA partly counters the pro-apoptotic effect of the C_2-ceramide by reducing the cytosolic release of HtrA2/Omi and the dephosphorylation of ERK1/2. Additionally, pretreatment with AA (0.01–100 nmol/L) improves the survival of human neuroblastoma SH-SY5Y cells against H_2O_2- or rotenone-induced injury [17] and glutamate-induced toxicity [16]. H_2O_2 or rotenone contributes to cell death via oxidative stress, while glutamate causes excitotoxicity under the overwhelming of intracellular Ca^{2+} concentration. However, in our present study, AA (1 μmol/L) did not show any protective effect against cholesterol-induced cytotoxicity in human neuroblastoma SH-SY5Y cells. This may be attributed to the different underlying mechanisms among these methods utilized to provide cell death. It has been reported that 20–80 μmol/L cholesterol can cause fetal rat enterocytes and also IEC-6 cells to be apoptosized via pronounced DNA fragmentation [20]. Furthermore, cholesterol at the concentration of 50 μmol/L can enhance gastric cancer cell death both by apoptosis and autophagy via caspase-3 cleavage, nuclear fragmentation, ATG accumulation or LC3 cleavage [21]. As our result reveals, 1 μmol/L AA has the propensity to slightly recover cell viability of cytotoxic human neuroblastoma SH-SY5Y cells, induced by cholesterol, which implies that AA is involved in the downstream processes of this cytotoxic mechanism attributable to cholesterol.

Although our study is a preliminary study, it does, however, confirm that a high cholesterol environment might be harmful to the cells and also suggests that asiatic acid has a recovery effect against cholesterol-induced cytotoxicity. This recovery effect cannot be conclusively stated due to the short-time period of our observations. Thus, further studies being undertaken will extend the post-treatment period and thereby further identify the mechanisms which lead to cholesterol-induced cytotoxicity.

Conflict of interest statement

We declare that we have no conflict of interest.

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