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Original Article

Neuroprotective Effects of Scopoletin on Neuro-damage caused by Alcohol in Primary Hippocampal Neurons

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Excessive drinking of alcohol is known to be one of the main causes of various neurological diseases, such as Alzheimer's disease. Scopoletin is known to have anti-inflammatory and antioxidative properties, and to protect nerve cells. This study examined whether scopoletin inhibits the alcohol-induced apoptosis of primary hippocampal neurons, and how scopoletin regulates several factors associated with the caspase-mediated pathway. To achieve this, the cell viability and apoptosis rate of primary hippocampal neurons were measured by Cell Counting Kit-8 and flow cytometry, respectively. Apoptosis-related protein expressions (Bax, Bid, caspase-3, caspase-9, and Poly (ADP-ribose) polymerase (PARP)) were analyzed by Western blotting, and the ANOVA method was used to confirm the significance of the measured results. As a result, scopoletin inhibited the expressions of alcohol-induced apoptosis and apoptosis-related proteins in primary hippocampal neurons. These results suggest that down-regulation of Bid, Bax, and cleaved caspase-9 expression induced by scopoletin down-regulates the expression of cleaved caspase-3, inhibits the expression of cleaved PARP, and finally, inhibits mitochondrial apoptotic pathways. The study suggests that scopoletin is worth developing as a candidate for neuroprotective agent.

Key Words: Apoptosis, Scopoletin, Primary hippocampal neuron, Bid, Bax, Caspase-9, Caspase-3, PARP

INTRODUCTION

When nerve cells in the brain die or do not function normally, memory and behavioral changes occur, and this is called dementia. Alzheimer's disease (AD), which is the most common dementia, is a degenerative brain disease. It is characterized by memory and cognitive ability decline (Barker et al., 2002; Kalaria et al., 2008; Wilson et al., 2012). Therefore, the hippocampus responsible for the storage function of memory has attracted attention in studies related to AD (Zarow et al., 2005; Hollands et al., 2016; Moreno-Jiménez et al., 2019). Also, AD is characterized by tangles of nerve fibers, amyloid plaques, and nerve loss. Neuronal loss is a common pathway in the degenerative process of AD and can be caused by various factors, for example, amyloid-beta plaques, inflammation, and oxidative stress (Baloyannis, 2006; Spires et al., 2006; Padurariu et al., 2010; Ciobica et al., 2011).

AD is caused for a variety of reasons, which include environmental, genetic, and lifestyle factors (Kalaria et al., 2008; Barnes and Yaffe, 2011). Excessive apoptosis plays a

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role in many neurological diseases, which include AD (Su et al., 2001; Dickson, 2004; Das and Vasudevan, 2007). Alcoholism causes brain damage, including brain shrinkage, reduction in numbers of neurons, oxidative stress, mitochondrial damage, and apoptosis (Bonthius and West, 1990; Mann et al., 2001; Young et al., 2003; Han et al., 2005; Zhong et al., 2006). Recent studies have indicated that chronic alcohol consumption increases the expression of genes associated with amyloid-beta production. The accumulation of amyloid-beta can induce neuronal cell death through the caspase pathway (Takahashi et al., 2002; Kim et al., 2011; Han et al., 2017). Caspase-mediated apoptosis has been known to be a significant mediator of cell death in some neurodegenerative diseases (Li et al., 2008).

In this study, we used scopoletin, which is a coumarin that is isolated from several plant species. This compound plays many roles in health treatment, such as inflammation, rheumatic pains, leprosy, and cardiovascular and neuromuscular actions (Carpinella et al., 2005; Ding et al., 2008; Gnonlonfin et al., 2012; Pandy et al., 2014). According to recent research, Morinda citrifolia fruit extract (MCE) with scopoletin mitigates skeletal muscle damage through anti-apoptosis (Narasimhan et al., 2016). Scopoletin suppresses apoptosis in the neuronal cell through the prevention of oxidative stress (Narasimhan et al., 2019). It also inhibits acetylcholinesterase enzyme (AChE) that could play a key role in accelerating amyloid-beta plaque deposition (Inestrosa et al., 1996; Rollinger et al., 2004).

AD is a degenerative brain disease, so neuronal cells are used in research. Among them, primary neuronal cells are they can produce results close to *in vivo* research. In general, when performing experiments related to AD, inflammation, and apoptosis caused by substances such as amyloid-beta and alcohol are measured (Calissano et al., 2009; Fonseca et al., 2009; Scuderi et al., 2014). We used a model of alcohol-induced apoptosis in the hippocampal neurons most associated with AD.

In this present study, we assumed that scopoletin has a protective effect on apoptosis to primary hippocampal cells caused by alcohol, and investigate how scopoletin regulates several factors that are related to the caspase-mediated pathway. So, we measured cell viability, cell apoptosis rate, and the level of protein (Bax, Bid, Caspase-3, Caspase-9, and Poly (ADP-ribose) polymerase (PARP)) expression associated with apoptosis in the primary hippocampal neuron. The purpose of this study is to clarify the protection of scopoletin against alcohol-induced apoptosis of primary hippocampal neurons. This may provide a theoretical basis for scopoletin for clinical treatment for neuro-damage caused by alcohol.

MATERIALS AND METHODS

Materials

Scopoletin, Dimethyl sulfoxide (DMSO), and Accumax^{TM} solution were bought from Sigma-Aldrich (St. Louis, MO, USA). Anti-Bax, anti-caspase-9, anti-caspase-3, anti-PARP, anti-mouse IgG-horseradish peroxidase (HRP) conjugate, anti-rabbit IgG HRP antibodies, and lysis buffer were purchased from Cell Signaling Technology (Beverly, MA, USA). β-actin was bought from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bid was purchased from GeneTex (Irvine, CA, USA). Polyvinylidene difluoride (PVDF) membrane and Enhanced chemiluminescence (ECL) solution were purchased from GE Health Care (Chalfont St. Giles, Buckinghamshire, UK). Primary hippocampal neurons from Sprague-Dawley rat were obtained from KOATECH (Gyeonggi-do, Korea). Neuronal basal media, B-27 Supplement, Penicillin (100 U/mL), Streptomycin (100 μg/mL), GlutaMAX, and Sodium pyruvate were purchased from Gibco (Gaithersburg, MD, USA). Cell viability was measured using Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). To measure primary hippocampal neurons apoptosis, FITC annexin V apoptosis Detection Kit (BD Bioscience, Franklin Lakes, NJ, USA) was used.

Cell culture

Primary hippocampal neurons were prepared from Sprague-Dawley rat embryos at embryonic day 21. Brains were removed after decapitation, meninges stripped, and the collected hippocampus incubated with Accumax^{TM} solution at 37℃ CO_2 incubator for 15 min. Afterwards, brain tissue was washed with HBSS three times, and dissociated by pipetting into starter media (Neuronal basal media with B27,

glutaMAX, sodium pyruvate, penicillin-streptomycin). Cells were counted by hemocytometer, and seeded at 3 $\times$ 10^5 in 6-well plates. Before use, the plates were pretreated with poly-L-lysine (0.5% w/v in autoclaved TDW) at 37℃ for at least 1 h. Cultures were kept at 37℃ and 5% CO_2, and half of the medium was replaced twice a week. All animal experiments were carried out according to the guidelines of the Institutional Animal Care and Use Committee of Konyang University (P-20-08-E-01) (Daejeon, South Korea).

Cell viability analysis

Cell viability was measured using the Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). The cells were seeded into 96-well plates (1 $\times$ 10^4 cells), and cultured for 14 days. Afterwards, cells were pretreated for 3 h with various concentrations of scopoletin, and treated without or with 400 mM alcohol. After incubation for 24 h, 10 μL of CCK-8 solution was added to each well, and the cells incubated for 3 h. Scopoletin was dissolved in a final concentration of ≤ 0.1% with DMSO. DMSO is not affected the results (data not shown).

The fluorescence intensity of the plates was then measured at 450 nm by SpectraMax iD3 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Western blot analysis

Cells were lysed on ice in RIPA buffer for 20 min. They were then centrifuged, and the supernatant containing the protein (5 μg) was mixed with sample buffer, and boiled at 95℃ for 5 min. Protein concentrations were measured using DC™ Protein Assay (Bio-Rad Laboratories, USA). The samples were isolated by 12% SDS-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene fluoride (PVDF) membranes. After blocking with 1× blocking buffer (BioFact Biofactory, Daejeon, Korea) at room temperature for 1 h, the membranes were incubated overnight with primary antibodies at 4℃. Next day, membranes were incubated with secondary antibodies, and detected by ECL. The blots were analyzed using ImageQuant™ LAS 500 (GE Healthcare, Chicago, IL, USA). The dilutions for anti-caspase -9, anti-caspase-3, anti-Bid, anti-Bax, anti-PARP, β-actin, and anti-mouse IgG-horseradish peroxidase (HRP) conjugate, anti-rabbit IgG HRP antibodies were 1:2,000. Scopoletin was dissolved in a final concentration of ≤ 0.1% with DMSO. DMSO is not affected the results (data not shown).

Detection of apoptosis

Apoptosis was detected using FITC annexin V apoptosis Detection Kit (BD). The cells were seeded into 6-well plates (3 $\times$ 10^5), and cultured for 14 days. Next, cells were pretreated for 3 h with various concentrations of scopoletin, and were treated without or with 400 mM alcohol. After incubation for 24 h, the cells were washed using PBS buffer, and then resuspended in binding buffer. Cells were next incubated with FITC Annexin V and Propidium iodide (PI) for 15 min in the dark. Binding buffer was added, and cell apoptosis was analyzed by BD Accuri™ C6 Plus Flow Cytometer (BD Bioscience, Franklin Lakes, NJ, USA). Scopoletin was dissolved in a final concentration of ≤ 0.1% with DMSO. DMSO is not affected the results (data not shown).

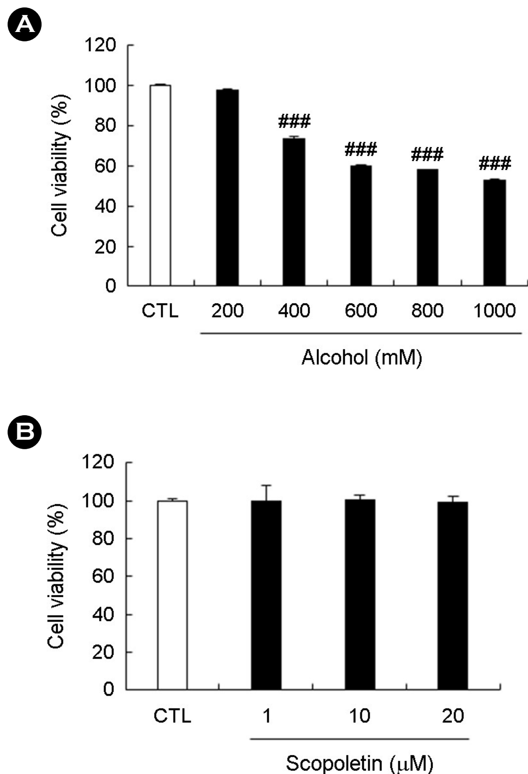
Statistical analysis

The experimental results are presented as the mean ± S.D., and statistical analysis was performed by one-way analysis of variance (ANOVA). The results with P-value < 0.05 were considered statistically significant.

RESULTS

Effects of scopoletin on cell viability in alcohol-treated primary hippocampal neurons

Alcohol induces cell death in the neuron in a concentration-dependent manner (Fig. 1A). Therefore, reducing the neuron cell death by scopoletin might be protective against alcohol-induced neuro-damage. When primary hippocampal neurons were exposed to 400 mM alcohol, the cell survival rate was decreased to 65% over the control (100%), whereas scopoletin-enhanced cell viability increased to 82% at 20 μM (Fig. 1C). These results show that primary hippocampal neuron death is suppressed by scopoletin in a concentration-dependent manner. Additionally, scopoletin is confirmed to have a non-cytotoxic effect in a dose-dependent manner (Fig. 1B).



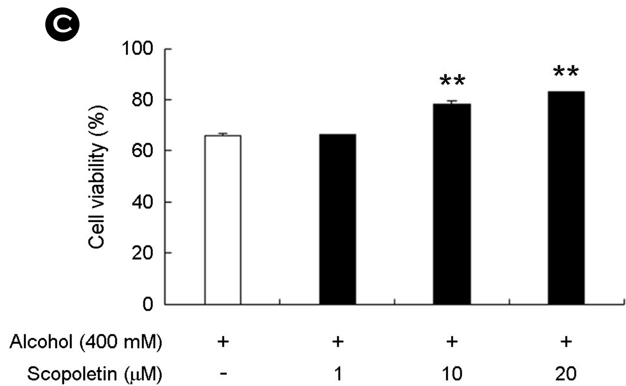


Fig. 1. Effects of scopoletin on cell viability in alcohol-treated primary hippocampal neuron. (A) Effect of alcohol on primary hippocampal neurons. (B) Effects of the scopoletin on primary hippocampal neurons. (C) Effects of scopoletin on 400 mM alcohol-treated primary hippocampal neurons. The cells were seeded into 96-well plates (1×10^4 cells). Scopoletin was pretreated in various concentrations. After 3 h, 400 mM alcohol was treated for 24 h, and 10 μL of CCK-8 reagent was added to each well. The 96-well plates were read by SpectraMax iD3 microplate reader (Molecular Devices, CA, USA) at 450 nM. Data are presented as mean ± S.D. (n=3). $^{###}P<0.001$ compared with untreated cells; $^{**}P<0.01$ compared to alcohol-treated cells.

Effects of scopoletin on Bid, Bax, caspase-9, caspase-3, and PARP levels in alcohol-treated primary hippocampal neurons

Alcohol is known to induce neuronal cell apoptosis. When primary hippocampal neurons were treated with alcohol for 24 h, the level of the apoptosis proteins Bid and Bax were significantly increased by 362 and 155%, respectively, compared to those of the control. In contrast, pre-treatment with 1, 10, and 20 μM of scopoletin was significantly reduced (Figs. 2A and 2B). Furthermore, 1, 10, and 20 μM of scopoletin reduced the level of apoptosis protein cleaved caspase-9 and cleaved caspase-3 on the alcohol-treated primary hippocampal neurons. Once primary hippocampal neurons were treated with alcohol for 24 h, the level of the apoptosis protein cleaved caspase 9 and cleaved caspase 3 were increased by 270 and 272%, respectively, compared to those levels of control. Contrariwise, pre-treatment with 1, 10, and 20 μM scopoletin significantly reduced these levels in a dose-dependent manner (Figs. 2C and 2D). Also, alcohol-treated neurons increased cleaved PARP. When primary hippocampal neurons were treated with alcohol for 24 h, apoptosis protein cleaved PARP level was increased, compared to those of the control (127%). In contrast, pretreatment with 1, 10, and 20 μM of scopoletin reduced levels in a dose-dependent manner (Fig. 2E).

Effects of scopoletin on apoptosis in alcohol-treated primary hippocampal neurons

Fig. 3 shows that when treated by 400 mM alcohol, the percentage of annexin V + PI loaded apoptotic cells increased to 6.1%. However, scopoletin pre-treatment at 1, 10, and 20 μM significantly reduced cell apoptosis in a dose-dependent manner at 5.1, 3.5, and 3.4%, respectively. The results suggest that the anti-apoptosis activity of scopoletin in alcohol-treated primary hippocampal neurons was due to the reduction of apoptotic cells.

DISCUSSION

Rat primary hippocampal neurons are frequently used in experiments related to neuronal apoptosis and neurodege-

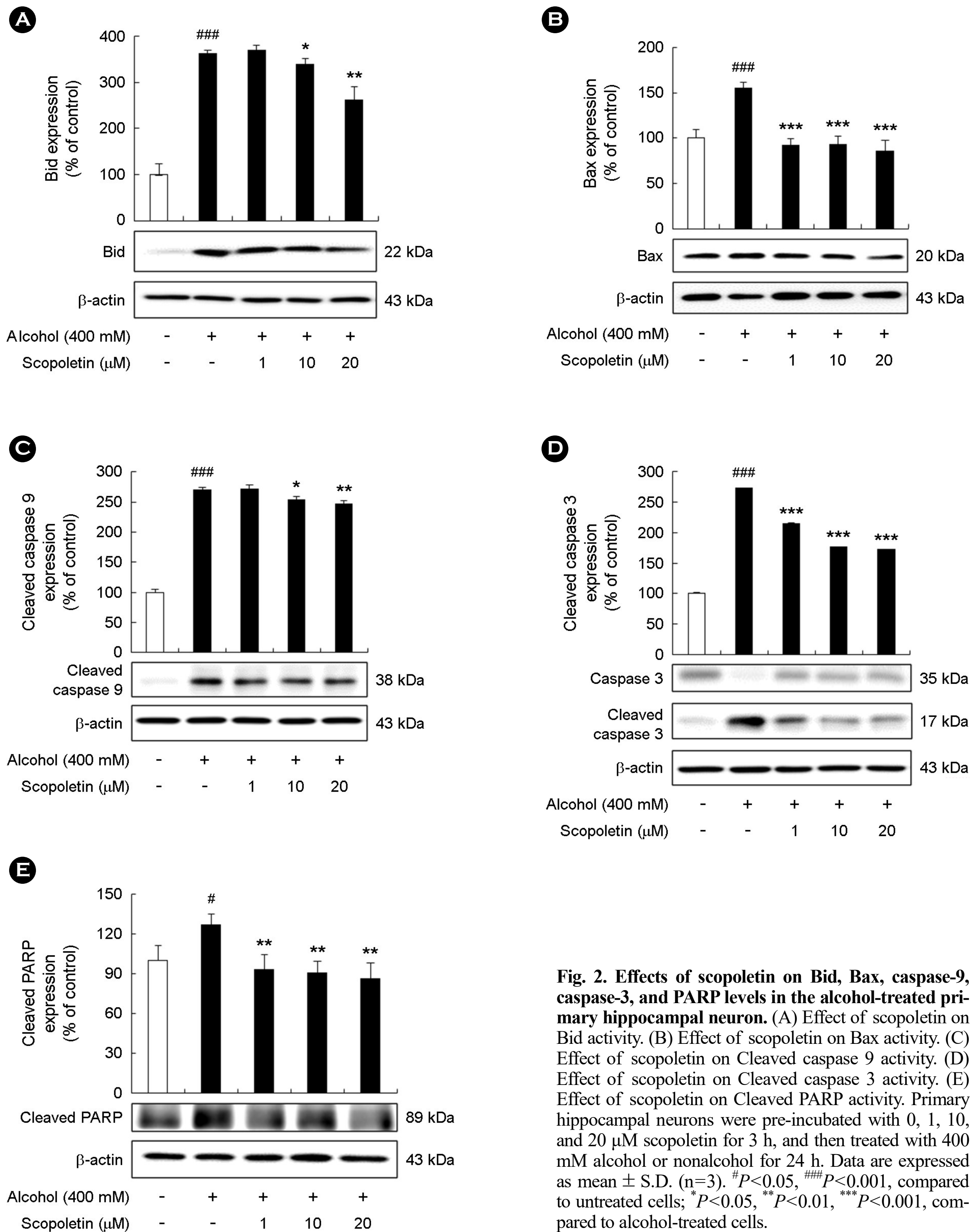


Fig. 2. Effects of scopoletin on Bid, Bax, caspase-9, caspase-3, and PARP levels in the alcohol-treated primary hippocampal neuron. (A) Effect of scopoletin on Bid activity. (B) Effect of scopoletin on Bax activity. (C) Effect of scopoletin on Cleaved caspase 9 activity. (D) Effect of scopoletin on Cleaved caspase 3 activity. (E) Effect of scopoletin on Cleaved PARP activity. Primary hippocampal neurons were pre-incubated with 0, 1, 10, and 20 μM scopoletin for 3 h, and then treated with 400 mM alcohol or nonalcohol for 24 h. Data are expressed as mean ± S.D. (n=3). $^{\#}P<0.05$, $^{\#\#\#}P<0.001$, compared to untreated cells; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, compared to alcohol-treated cells.

nerative disease (Tamatani et al., 1999; Murphy et al., 2000; Zhao et al., 2004). One of the main principles of alcohol that damages the central nervous system and kills neurons is to increase reactive stress species (ROS) and cause mito-

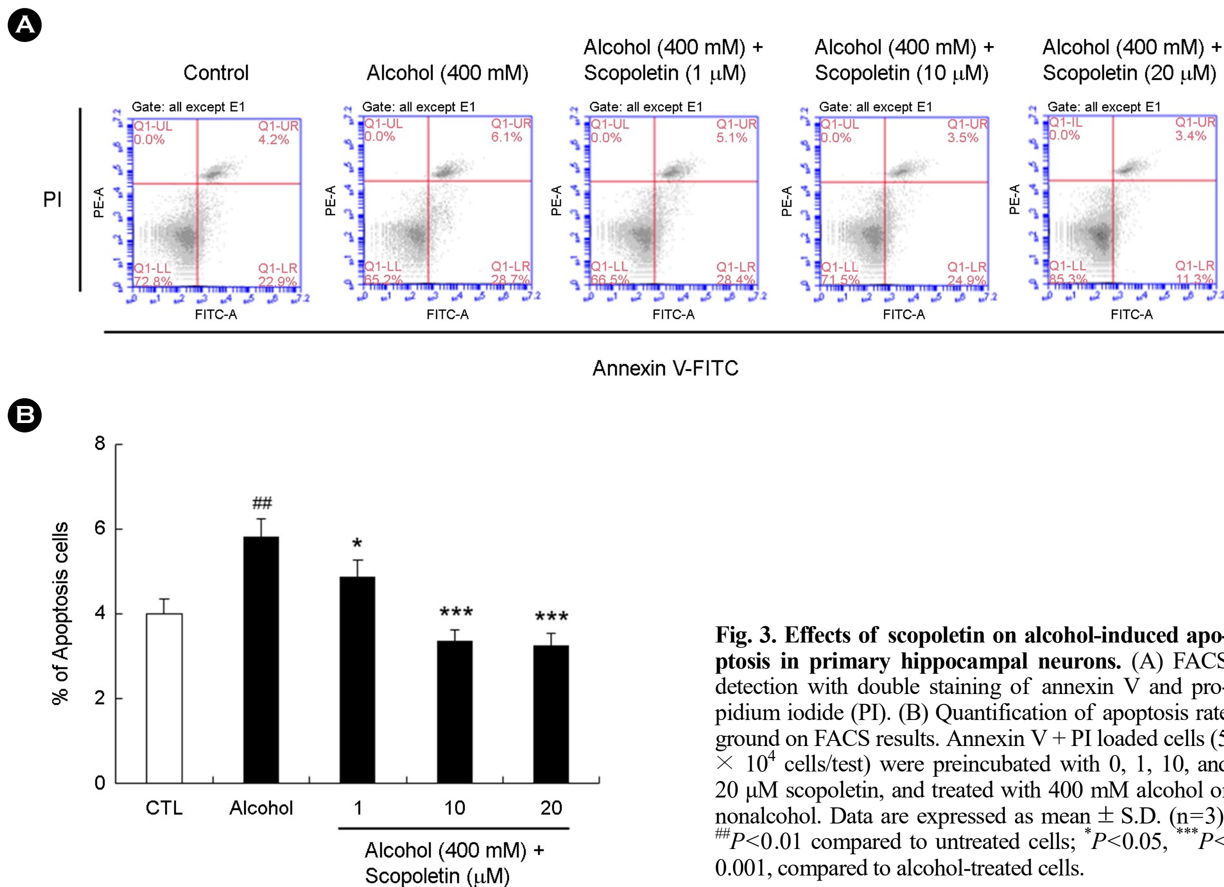


Fig. 3. Effects of scopoletin on alcohol-induced apoptosis in primary hippocampal neurons. (A) FACS detection with double staining of annexin V and propidium iodide (PI). (B) Quantification of apoptosis rate ground on FACS results. Annexin V + PI loaded cells (5×10^4 cells/test) were preincubated with 0, 1, 10, and 20 μM scopoletin, and treated with 400 mM alcohol or nonalcohol. Data are expressed as mean ± S.D. (n=3). $^{\#\#}P<0.01$ compared to untreated cells; $^{*}P<0.05$, $^{***}P<0.001$, compared to alcohol-treated cells.

chondrial DNA damage, leading to neuronal cell death and necrosis (Fadda and Rossetti, 1998). According to recent studies, many compounds extracted from natural products play an important role in protection against damage to neuronal cells. Among them, scopoletin has been known to have a protective effect on neuronal damage against oxidative stress (Inestrosa et al., 1996). Therefore, it can be beneficial for alcohol-induced neurotoxicity. However, the molecular mechanisms of scopoletin on alcohol-induced neuronal damage have not yet been explained.

To investigate the neuroprotective effect of scopoletin on alcohol-induced apoptosis in primary hippocampus neurons, preliminary experiments in this study were conducted to induce cell death of primary hippocampus neurons using 400 mM alcohol. For cell viability, the optimum dose of scopoletin was experimented with, and cell apoptosis measured. Expression of Bax, Bid, cleaved caspase-9, cleaved caspase-3, and cleaved PARP further clarified the mechanisms. The results show a marked decrease in cell death and cell viability in primary hippocampal neurons treated with 400 mM alcohol. All these results prove that the model is successful. In contrast, treatment with scopoletin presented the inhibitory effect of primary hippocampal neurons on alcohol-induced cell death. The concentration of scopoletin was conducted up to 30 μM (Narasimhan et al., 2019), and the experiment showed the most effect at 20 μM. Oxidative stress leading to mitochondrial dysfunction plays a significant role in the alcohol-induced neuronal damage mechanism, and one of the major factors of neuronal cell death is the potential loss of mitochondrial membrane (Fadda and Rossetti, 1998). Thus, when scopoletin inhibits the apoptosis of primary hippocampal neurons, it may be the result of protecting mitochondrial membrane from potential loss. The results of this experiment show that alcohol-treated primary hippocampal neurons increased the expression of cleaved caspase-3, whereas scopoletin pretreated neurons effectively inhibited

cleaved caspase-3.

Bcl-2 family proteins play an important role in cell apoptosis. When apoptosis occurs through the extrinsic pathway death receptor, activated caspase-8 activates BH3 interacting-domain death agonist (Bid), which a member of the Bcl-2 protein family. Activated Bid enters the intrinsic pathway, and activates Bcl-2-associated X protein (Bax); activated Bax can penetrate into mitochondrial outer membranes, which leads to apoptosis. So, when confirming the apoptosis of caspase-3 expression, it is effective to confirm the Bid and Bax levels.

In the present study, scopoletin inhibited Bid and Bax, and suppressed caspase-9 cleavage by caspase-9 activation. Thus, the expression of cleaved caspase-3 and the expression of cleaved PARP were suppressed. This indicates that scopoletin inhibits caspase-3 by inhibiting Bid, Bax, and caspase-9, and reduces PARP cleaved by caspase-3. Apoptosis has extrinsic and intrinsic pathways. Mitochondria play an important role in intrinsic pathways apoptosis. Cytochrome c exits between the apoptotic pores formed by Bax, bak, etc., migrates to the cytoplasm and binds to apaf1 and caspase-9 to form apoptosomes. We have experimentally confirmed that scopoletin has an anti-apoptotic effect through the intrinsic pathway. In alcohol-induced apoptosis, scopoletin initiates the anti-apoptosis effect by inhibiting the Bid that links extrinsic and intrinsic pathway apoptosis. Also, by inhibiting Bax, apoptotic pore formation is suppressed, and caspase-9 activity is suppressed to suppress apoptosome formation. It inhibits the activity of caspase-9 and inhibits the activity of caspase-3 by the sequential cascade. These results suggest that down-regulations of Bid, Bax, and caspase-9 activation by scopoletin suppress caspase-3 activation, cleavage of PARP, and finally inhibit mitochondrial apoptosis pathways. This shows the protective mechanism of scopoletin on alcohol-induced apoptosis in primary hippocampal neurons. This data was obtained from *in vitro* experiments, and it is necessary to examine the scopoletin effect on alcohol-induced neurotoxicity rodent models. Nevertheless, the study presents novel evidence that scopoletin can be applied as a candidate for neuroprotection.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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