Curcuma longa Linn extract suppresses neuronal apoptosis induction by sevoflurane via activation of the ERK1/2 pathway

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Abstract

Purpose: To investigate Curcuma longa Linn against neuronal damage induced by exposure to sevoflurane during surgical procedures.

Methods: A sealed box made of transparent glass was used for anaesthetic exposure of neurons. The neurons were exposed to Curcuma longa Linn at doses of 1.5, 3, 6 and 12 µM prior to viability assessment using MTT assay. The effect of Curcuma longa Linn treatment on protein expression was determined using western blotting.

Results: Sevoflurane exposure led to significant and time-dependent reductions in neuronal proliferation, when compared to unexposed cells (p < 0.05). Curcuma longa Linn at doses of 1.5, 3, 6 and 12 µM significantly decreased sevoflurane-mediated neuronal apoptosis. It reduced cleaved caspase-3 and Bax levels in neurons. However, the Curcuma longa Linn-mediated inhibition of sevoflurane-induced neuronal apoptosis was significantly suppressed by VPC23019 (p < 0.05). The p-ERK1/2 level was dose-dependently up-regulated in neurons exposed to sevoflurane on treatment with Curcuma longa Linn. Moreover, VPC23019 reversed the upregulatory effect of Curcuma longa Linn on p-ERK1/2 expression in sevoflurane-exposed neurons (p < 0.05).

Conclusion: Curcuma longa Linn reversed sevoflurane-induced neuronal apoptosis by elevating p-ERK1/2 expression. Therefore, Curcuma longa Linn exerts inhibitory effect on anaesthesia-induced apoptosis in neurons, and may be useful for the treatment of this condition.

Keywords: Anesthesia, Neurotoxicity, Sphingomyelin, Apoptosis, Curcuma longa

INTRODUCTION

Anaesthesia is most frequently used in surgical procedures for the convenience of patents, and also in response to patients’ demand [1,2]. However, increased application of anaesthesia has led to many complications in patients, including cognitive dysfunction following surgery [3]. Due to these complications, the effect of anaesthetic compounds on the nervous system, especially their effects on memory and cognition potential in children and infants, have been investigated [4]. The most frequently used anaesthetic compound for surgeries is

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sevoflurane, due to its stable hemodynamics, quick patient recovery, and negligible deleterious impact on the kidneys and liver [5]. Studies have found that sevoflurane inhibits proliferative potential of cortical progenitor cells and promotes neuronal death in cortex of CNS [6]. Thus, sevoflurane causes memory impairment and loss of cognition in new-born animal models [9]. It has been demonstrated that sevoflurane induces toxicity and neuronal apoptosis which subsequently result in cognitive impairment [12,13]. Thus, there is need for prevention of sevoflurane-induced neurotoxicity so as to inhibit memory loss. Sphingomyelin is an important part of oligodendrocytes and myelin covering in the CNS [7]. The conversion of sphingomyelin to ceramide by phospholipase C is followed by its transformation to sphingosine by the enzyme ceramidase [8]. Phosphorylation of sphingosine leads to its conversion to sphingosine-1-phosphate (S1P) in a reaction catalyzed by sphingosine kinase (SphK) [9]. The survival and proliferation of neurons are associated with the activation of S1P signal pathway [10]. Neuraminic acid treatment has been demonstrated to upregulate the S1P pathway, thereby targeting apoptosis in PC12 cells [10]. Impaired transmission of the sphingosine kinase 1/S1P signal pathway causes disruption in neuronal cell survival and growth, resulting in defective neural progenitor cells [11].

**Curcuma longa** Linn (CL) grows in tropical and subtropical regions world-wide [12]. The plant has traditional medicinal importance with respect to healing of wounds, and treatment of infectious fever and liver diseases [12]. Moreover, it has anti-inflammatory potential [13], hepatoprotective property [13] and anti-arthritic uses. Present study investigated *Curcuma longa* Linn against neuronal damage induced by exposure to sevoflurane during surgical procedures.

**EXPERIMENTAL**

**Preparation of hippocampal neurons**

Sprague-Dawley rats (20 day old; body weight = 48 g) were provided by the Animal Centre of Beijing (Beijing, China). Temperature was maintained at 22 ± 2 °C, relative humidity 40 % and day/night durations were controlled to 12- h/12-h. Laboratory feed and water was made freely available to the rats in animal center. The procedures used were carried out in aortane with international guidelines [14]. Following sacrifice through decapitation, the skin around each rat skull was carefully removed, followed by separation of the cerebral hemispheres. The excised tissues were put in petri dishes, and the cortex was isolated to expose and remove the hippocampus. Following washing in Hank’s D solution, the tissues were subjected to centrifugation at 200 x g for 10 min at 4 °C. Decantation of supernatant was followed by digestion with 10 μL of protease for 15 - 20 min. Thereafter, DMEM and 10 % FBS were added, and the cells were subjected to filtration through 200-mesh size copper filter. Centrifugation and DMEM addition were followed by culture of the single cell suspension in culture flasks at 37 °C.

**Treatment of neurons**

Neurons seeded at 2 x 10^6 cells/ well distribution in 96- well plates were cultured for 24 h. Thereafter, the neurons were incubated overnight for 24 h with *Curcuma longa* Linn at doses of 1.5, 3, 6 and 12 μM; VPC23019 (0.5 μmol/L) and U0126 (5 μmol/L). Study approval by obtained from the Ethics Committee for Animal Use, The First People’s Hospital of Fuyang District (Fuyang, China; approval no. = UU/002/18). All experiments were conducted according to the guidelines issued by the National Institute of Health China.

**Exposure to anaesthesia**

A sealed box made of transparent glass was used for exposure of the neurons to anaesthesia. The box contained 100 g of soda lime at the bottom, and apertures on the lateral sides were connected to anaesthesia machine (Dräger Fabius® GS Premium, Germany) using threaded pipe. The primary hippocampal neurons were distributed in Matrigel basement coated 24- well plates at a density of 2 x 10^5 cells/well in neurobasal medium. The neurons were exposed to *Curcuma longa* Linn at doses of 1.5, 3, 6 and 12 μM, after which they were subjected to tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylytetrazolium bromide (MTT) assay.

**Cell viability assay**

The neuronal cells were seeded in 96-well plates at a density of 2 x 10^5 cells/well, and were cultured in a 5% CO₂ incubator at 37°C. The neurons were exposed to *Curcuma longa* Linn at doses of 1.5, 3, 6 and 12 μM; and 0.5 μmol/L VPC23019 for 24 h. Thereafter, 20 μL of MTT solution (5 mg/mL) was added to each of the wells, and incubation was continued for additional 4 h. The formazan crystals formed were dissolved by addition of 150 μL of DMSO to each well. The absorbance of the solution in each well was read at 488 nm in an enzyme-linked immunodetector.
Western blot analysis

The medium was discarded from the plates and the cells were washed, agitated and subsequently lysed in radioimmunoprecipitation buffer for 45 min. The lysate was transferred with a pipette into a centrifuge tube and centrifuged at 1,200 x g for 10 min at 4 °C. The protein content of each lysate was estimated using BCA assay. Then, 50-μg samples were resolved on SDS-PAGE, followed by transfer onto PVDF membranes which were blocked using 5% non-fat milk solution. Thereafter, the membranes were incubated overnight with the primary antibodies anti-Bax, anti-caspase-3, anti-Bcl2, anti-p-ERK, anti-ERK and anti-β-actin at 4 °C. Then, the membranes were washed and incubated for 2 h with goat anti-rabbit IgG secondary antibody at 37 °C. The proteins were visualized using ECL system connected to Odyssey far-infrared fluorescence system (LI-COR Biosciences, USA).

Statistical analysis

Data are expressed as mean ± SD of triplicate measurements. Statistical analysis of the data was made using SPSS 19.0 statistical software. One-way analysis of variance (ANOVA) and post-hoc test were used for comparison of data. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Sevoflurane inhibits neuron viability

Exposure of neurons to sevoflurane for 2, 4, 8, 16 and 32 h was followed by evaluation of cell proliferation (Figure 1). There was a significant and time-dependent reduction in the proliferation of neurons exposed to sevoflurane, relative to unexposed neurons ($p < 0.05$). Neuronal proliferation was reduced to 91, 83, 70, 56 and 49 %, by sevoflurane at 2, 4, 8, 16 and 32 h, respectively.

Curcuma longa Linn prevented apoptosis by sevoflurane in neurons

Curcuma longa Linn treatment prevented sevoflurane-mediated activation of apoptosis in neurons in a dose-dependent manner (Figure 2). Significant decreases in sevoflurane-mediated apoptosis by Curcuma longa Linn treatment were observed at doses of 1.5, 3, 6 and 12 µM. At a dose of 12 µM, Curcuma longa Linn treatment suppressed sevoflurane-mediated neuronal apoptosis to the lowest level.

Figure 1: Effect of sevoflurane on proliferation of neurons. Neurons exposed to 3 % sevoflurane for 2, 4, 8, 16 and 32 h, and unexposed neurons were examined for proliferative changes using MTT assay. *$p < 0.05$, **$p < 0.02$, vs. sevoflurane group

Curcuma longa Linn inhibited sevoflurane-mediated expressions of apoptotic proteins in neurons

Treatment with Curcuma longa Linn reduced levels of cleaved caspase-3 and Bax in neurons exposed to sevoflurane (Figure 3). The levels of Bcl-2 in neurons exposed to sevoflurane were elevated on treatment with Curcuma longa Linn.

Figure 3: Curcuma longa Linn suppresses expressions of apoptosis-related proteins. (A) Treatment of neurons with Curcuma longa Linn at doses of 1.5, 3, 6 and 12 µM after sevoflurane exposure was followed by Western blotting. (B) Protein band quantification levels. *$p < 0.05$; **$p < 0.02$, vs. sevoflurane group
VPC23019 reversed *Curcuma longa* mediated anti-apoptotic effect in neurons exposed to sevoflurane

*Curcuma longa* Linn-mediated antiapoptotic effect in neurons exposed to sevoflurane was markedly suppressed by VPC23019 (Figure 4). Moreover, VPC23019 upregulated cleaved caspase-3 cleavage and Bax level in *Curcuma longa* Linn-treated and sevoflurane-exposed neurons. The Bcl2 level in *Curcuma longa* Linn-treated and sevoflurane-exposed neurons was effectively reduced by VPC23019.

Figure 4: Effect of VPC23019 on *Curcuma longa* Linn-mediated inhibition of apoptosis. (A) Apoptosis in untreated, sevoflurane-exposed, *Curcuma longa* Linn-treated + sevoflurane exposed, and *Curcuma longa* Linn-treated + sevoflurane-exposed + VPC23019-treated groups of neurons, as measured using MTT assay. (B) Protein levels. *P < 0.05, **p < 0.02, vs. sevoflurane group

U0126 reversed *Curcuma longa* Linn-mediated inhibition of apoptotic changes due to sevoflurane

Inhibition of apoptosis by *Curcuma longa* Linn in neurons exposed to sevoflurane was effectively suppressed by U0126 (Figure 5). Compared to *Curcuma longa* Linn-treated neurons, apoptosis was significantly increased by U0126 in *Curcuma longa* Linn-treated and sevoflurane-exposed neurons. Moreover, U0126 elevated cleaved caspase-3 and Bax levels in *Curcuma longa* Linn-treated and sevoflurane-exposed neurons, relative to *Curcuma longa* Linn-treated neurons. In addition, U0126 suppressed Bcl2 expression in neurons treated with *Curcuma longa* Linn, relative to neurons treated with *Curcuma longa* Linn. and sevoflurane.

Curcuma longa Linn upregulated p-ERK1/2 expression

In neurons exposed to sevoflurane, there was a marked reduction in p-ERK1/2 protein, when compared to control cells (Figure 6). In contrast, p-ERK1/2 protein was markedly and dose-dependently upregulated by *Curcuma longa* Linn in neurons exposed to sevoflurane. However, VPC23019 reversed the up-regulatory role of *Curcuma longa* Linn on p-ERK1/2 expression in neurons exposed to sevoflurane.

Figure 5: Effect of U0126 on *Curcuma longa* Linn-mediated inhibition of apoptosis. (A) Apoptosis in untreated, sevoflurane-exposed, *Curcuma longa* Linn-treated + sevoflurane-exposed + U0126-treated groups of neurons, as measured using MTT assay. (B) Protein levels of apoptotic factors in the various groups of neurons. *P < 0.05, **p < 0.02, vs. sevoflurane group

DISCUSSION

Sevoflurane, an anesthetic compound used during surgeries, has been reported to induce apoptosis in neurons and subsequent impairment of memory and cognitive potential [15]. Sevoflurane-induced neuronal damage has been reported in Alzheimer's disease patients as well as in healthy people [16]. Many pathways associated with signal transduction such as NF-kB and S1P have been found to be involved in sevoflurane-induced neurological damage [17]. In agreement with findings in previous studies, the present study has revealed that exposure of neurons to sevoflurane resulted in time-dependent suppression of proliferation. Neuronal proliferation was reduced to 91, 83, 70, 56 and 49% by sevoflurane at 2, 4, 8, 16 and 32 h, respectively. Activation of sevoflurane-induced neuronal apoptosis was inhibited in a dose-
dependent manner on treatment with Curcuma longa Linn. It has been demonstrated that the sphingosine-1-phosphate (S1P) pathway enhances the growth and proliferation of neurons [18]. The activation of S1P1 by chemotherapeutics has been reported to mitigate induction of neuronal apoptosis by anaesthetic compounds [19]. Since S1P1 activation is involved in signal transductions required for survival of neurons, a study determined effect of VPC23019 (S1P1 antagonist) on Curcuma longa Linn-mediated suppression of apoptosis in neurons exposed to sevoflurane [10]. In the present study, Curcuma longa Linn-mediated inhibition of apoptosis in neurons exposed to sevoflurane was prevented significantly by VPC23019. Moreover, VPC23019 upregulated the expressions levels of cleaved caspase-3 and Bax in Curcuma longa Linn-treated and sevoflurane-exposed neurons. The Bcl2 level in Curcuma longa Linn-treated and sevoflurane-exposed neurons was effectively reduced by VPC23019. These data indicate that Curcuma longa Linn suppressed apoptosis in sevoflurane-exposed neurons by activation of the S1P1 pathway.

Anaesthetic compounds generally target ERK which is the major pathway involved in neuronal apoptosis [20, 21]. Neuronal viability is maintained by p-ERK1/2 during exposure to anaesthetic compounds [22]. In the present study, exposure to sevoflurane led to reduction in p-ERK1/2 protein in neurons, relative to unexposed neurons. The p-ERK1/2 level was promoted markedly in neurons exposed to sevoflurane by Curcuma longa Linn treatment. However, VPC23019 reversed the Curcuma longa Linn mediated enhancement in p-ERK1/2 expression in neurons exposed to sevoflurane.

CONCLUSION

Curcuma longa Linn extract reverses sevoflurane-induced apoptosis in neurons by upregulating the ERK1/2 phosphorylation. The S1P1 pathway in sevoflurane-exposed neurons is also enhanced by Curcuma longa treatment. These results suggest that the extract exerts protective effect against anaesthesia-induced apoptosis in neurons. Thus, it may be beneficial in the treatment of neuronal apoptosis induced by anaesthetics.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

CONTRIBUTION OF AUTHORS

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yao Yan and Ying Lou performed the experimental work, carried out the literature survey and analysed and compiled the data. Zhou Yu designed the study and wrote the manuscript. All authors read the paper thoroughly and approved it for publication.

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