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Regular paper

Synthesis of oxymatrine hydrazone and its preventive action against sevoflurane induced neuron damage through ERK pathway up-regulation

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Exposure of patients undergoing multiple surgeries to anesthetic compounds leads to harmful side effects such as memory loss and impaired cognition. The current study was aimed to synthesize and investigate the effect of oxymatrine hydrazone on neuronal toxicity induced by sevoflurane in rats. Incubation with oxymatrine hydrazone was followed by exposure to sevoflurane for 48 h and determination of proliferation by 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Apoptosis was detected by flow cytometry using Annexin V-FITC and propydium iodide staining. Western blot analysis was used for determination of changes in protein expression. Sevoflurane exposure significantly (P<0.05) reduced proliferation of neurons by activation of cell apoptosis. However, pretreatment of neurons with oxymatrine hydrazone prevented reduction of proliferative potential induced on exposure with sevoflurane. Pre-treatment of neurons with 5.0 μM doses of oxymatrine hydrazone significantly prevented apoptosis induction by sevoflurane. Moreover, oxymatrine hydrazone pretreatment inhibited BCL2 Associated-X (BAX) and cleaved caspase-3 levels induced by sevoflurane exposure in neurons. Phosphorylation of extracellular signal-regulated protein kinase (ERK1/2) and expression of BCL-2 in neurons exposed to sevoflurane were markedly promoted on pretreatment with oxymatrine hydrazone. Additionally, U0126 (ERK ½ activation inhibitor) treatment of sevoflurane exposed neurons inhibited promotion of ERK1/2 phosphorylation by oxymatrine hydrazone pre-treatment. In summary, cytotoxicity of sevoflurane in neurons was prevented on pretreatment with oxymatrine hydrazone. Pretreatment of sevoflurane exposed neurons with oxymatrine hydrazone inhibited apoptosis, suppressed BAX/caspase-3 and elevated BCL-2. Moreover, oxymatrine hydrazone pre-treatment promoted ERK1/2 phosphorylation in sevoflurane exposed neurons. Therefore, oxymatrine hydrazone has a great potential for prevention of neurotoxicity induced by sevoflurane.

Keywords: sevoflurane, neurotoxicity, alkaloid compound, memory loss, apoptosis

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Abbreviations: BAX, BCL2 Associated-X; U0126, ERK ½ activation inhibitor; DMEM, Dulbecco's modified Eagle medium; FBS, Fetal Bovine Serum; ERK1/2, Extracellular signal regulated protein kinase; MTT, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

INTRODUCTION

General anesthesia is commonly given to patients at the time of surgery because it does not cause pain and is comfortable (Wan Hassan *et al.*, 2018). However, repeated exposure of patients undergoing multiple surgeries to anesthetic compounds leads to harmful side effects such as memory loss and impaired cognition (Anderson, 2018). Toxicity caused by anesthetic chemicals in nervous system of people after exposure has been demonstrated by many reports (Schwartz, 2018; Moran, 2017). Studies have shown harmful impact of anesthetic chemicals on memory and aptitude of infants and children (Schwartz, 2018; Moran, 2017). Sevoflurane has been the preferably used anesthesia in the last decade due to its least toxic effect on kidneys, functioning of liver and rapid recovery of patients (Juodzente *et al.*, 2018). During preclinical investigations sevoflurane exposure was demonstrated to activate apoptotic pathway and subsequently exhibit adverse effect on cognitive functioning (Jang *et al.*, 2017; Lu *et al.*, 2018). Therefore, strategies to prevent toxic effect of sevoflurane on neuronal survival and inhibit nervous system damage are urgently required.

Compounds obtained from natural resources inhibit inflammatory responses and therefore have been used for treatment of such disorders (Jeyaseelan *et al.*, 2005; Kazemi *et al.*, 2018). Oxymatrine is a natural product obtained from an herbaceous plant, *Sophora flavescens* and encompasses alkaloid structure (Blanco *et al.*, 2005). The compound showed good anti-inflammatory potential and has been screened for multiple pharmacological properties (Blanco *et al.*, 2005). During pharmacological investigation oxymatrine showed significant anti-oxidant and hepato-protective activities and is currently being used to treat inflammation of liver, traumatic brain injury and acute pancreatitis (Ping *et al.*, 2011; Xu *et al.*, 2005). Other reports have demonstrated that oxymatrine effectively suppresses inflammation mediated by influenza A virus infection (Zhang *et al.*, 2013). In the current study, effect of oxymatrine hydrazone was investigated on neuronal toxicity caused by sevoflurane exposure in mice. Moreover, the study also tried to explain the mechanism underlying oxymatrine hydrazone mediated prevention of neuronal damage induced by sevoflurane.

EXPERIMENTAL

Chemicals and reagents

All the chemicals and reagents used in the present study including $CDCl_3$, dimethyl sulfoxide, ortho-hydroxy phenyl hydrazine, potassium carbonate, methanol

and dichloromethane were purchased from Sigma-Aldrich (Merck KGaA, USA).

Synthesis and characterization of oxymatrine hydrazone

In the present study oxymatrine (1 mmol, 500 mg) was taken in a dry and clean 100 ml RB flask containing 10 ml ethyl alcohol. To this flask, ortho-hydroxy phenyl hydrazine (1.2 mmol) and potassium carbonate (1 equivalent) base were added, and the mixture was stirred at room temperature for 7.5 h. Progress of the reaction between the reactants was monitored using thin layer chromatography. The crude product obtained was purified by column chromatography using methanol and dichloromethane (10:90%) to obtain the desired compound in 89% yield.

The product obtained was characterized using 1H NMR and 13C NMR spectral techniques (Bruker Instrumentation, Germany) and the spectra are provided in Table 1.

Table 1. Characterization of oxymatrine hydrazone

1H NMR ($CDCl_3$, 400 MHZ)	δ 6.87-7.23 (4H, m), 5.18 (1H, s), 3.45 (1H, s), 2.39-2.17 (2H, m), 2.46-2.56 (5H, m), 1.79-1.53 (10H, m), 1.46-1.13 (7H, m), 0.82-0.91 (1H, m)
13C NMR ($CDCl_3$, 101 MHZ)	δ 153.4, 147.9, 135.3, 125.2, 120.8, 118.5, 115.3, 68.7, 46.5, 40.2, 37.8, 32.3, 30.6, 29.1, 27.2, 27.0, 24.4, 21.7, 21.2
ESI-MS	375.41 [M + Na^+]

Animals

Total ninety Male Sprague–Dawley rats (weight, 40–45 g; age, 18 days) were supplied by The First Hospital of Qinhuangdao, China. The rats were maintained at 23±2°C, exposed to standard 12-h light/dark periods and allowed to access food and water freely. Experiments for animals were approved by the Ethics Committee, Yan'an University, China (Approval number YU/102/17). The animal experimental procedures were performed in accordance with the European law for animal experimentation.

Isolation of rat hippocampus

Anesthetization of the rats with sevoflurane was followed by their sacrifice using decapitation method to isolate the hippocampus (1). Briefly, the skin around head of rats was removed after sterilization and cranial cavity was opened up to expose the brain. After isolation of brain, hippocampus was isolated and then washed three times with Hank's-D solution. The tissues were crushed at 4°C, centrifuged for 15 min at 12000×*g* at 4°C and the supernatant isolated was discarded. The tissues after digestion with protease (10 µl) for 30 min were agitated for 10 min after regular interval of 5 min. Then, DMEM containing 10% fetal bovine serum was added to the tissues and cells were isolated by filtration through 200-mesh size copper screen. The isolated cells were subjected to centrifugation at 12000×*g* for 15 min at 4°C followed by transfer to culture flasks containing DMEM (16). The cells were cultured at 37°C under an atmosphere of 5% CO_2 in DMEM for till attaining logarithmic growth (Fig. 1).

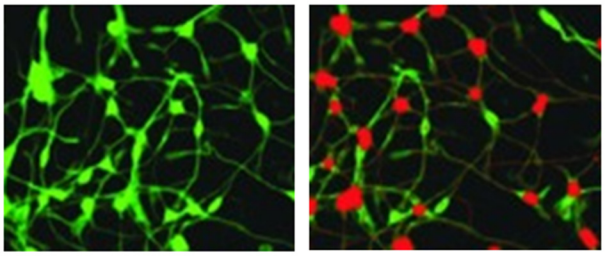

Figure 1. Cell neurons were cultured in 96-well plates at 2×10⁴ cells/ well density and visualized using Calcein AM.
Fluorescence microscopic images of the cells at 10× objective are shown in left panel. Neuronal bodies and outgrowth are seen in right panel as red dots and thin red lines, respectively.

Treatment strategy

The neuronal cells at 2×10⁵ cells/ well density were transferred to 96-well plates containing DMEM at 2×10⁵ cells per well density after attaining logarithmic growth. After culture for 24 h, the medium was changed by fresh medium mixed with oxymatrine hydrazone (0.5, 1.0, 2.0, 4.0 and 5.0 µM) or U0126 (5 µmol/l) and cells were incubated for 24 h. Pretreatment with oxymatrine hydrazone or U0126 was followed by exposure to sevoflurane for 6, 12, 24, 48 and 72 h. Sevoflurane (3%) was passed through threaded pipes into the sealed glass box containing ~100 g soda lime.

MTT assay

Neuronal cells were cultured at 2×10⁵ cells per well density in 96-well culture plates at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS; both Gibco; Thermo Fisher Scientific, Inc.). The cells were treated with 0.5, 1.0, 2.0, 4.0 and 5.0 µM doses of oxymatrine hydrazone and incubated for 72 h or 24 h at 37°C. Incubation with oxymatrine hydrazone was followed by exposure to sevoflurane for 48 h and determination of proliferation. At 72 h of sevoflurane exposure, 20 µl of MTT (5 mg/ml) solution was added to each well and neurons were incubated for 4 h more. Then dimethyl sulfoxide (120 µl) was added to each well and plates were kept in shaker for 20 min for solubilization of insoluble materials. Absorbance was measured for each well three times at 487 nm using microplate readers.

Apoptosis assay

Neuronal cells were pretreated for 24 h with 5.0 µM doses of oxymatrine hydrazone at 2×10⁵ cells per well density in 96-well plates at 37°C (1). After incubating, the cells were exposed to (3%) sevoflurane anesthesia for 72 h and subsequently washed in PBS. The cells were then stained with 5 µl Annexin V-FITC and 10 µl propydium iodide in 450 µl binding buffer for 15 min under conditions. Apoptosis was detected using flow cytometry (Cytomics FC 500; Beckman Coulter Inc., Miami, FL, USA).

Western blot analysis

The neurons pretreated with 5.0 µM doses of oxymatrine hydrazone for 24 h were subsequently exposed to (3%) sevoflurane anesthesia for 72 h and then washed with PBS. The cells were treated with RIPA buffer under ice-cold conditions for 50 min to

obtain lysate which was centrifuged at 12000×*g* for 20 min at 4°C. The supernatant obtained was subjected to estimation of protein concentration by bicinchoninic acid assay. Protein samples (30 μg) were resolved by loading on SDS-PAGE and subsequently transferred to PVDF membranes. The membrane non-specific sites were blocked by incubation with 5% non-fat dry milk for 2 h and then probed with primary antibodies at 4°C. Washing of membranes with TBS plus Tween® 20 three times was followed by incubation with goat anti-rabbit IgG secondary antibodies (Abcam, Cambridge, MA, USA) for 1 h. The blots were visualized using ECL reagent and fluorescence imaging system (LI-COR Biosciences, Lincoln, NE, USA). The primary antibodies used were against p-ERK (dilution 1:1000), caspase-3 (dilution 1:1000), BCL-2 (dilution 1:1000), BAX (dilution 1:1000) and β-actin (dilution 1:1000; Abcam, Cambridge, MA, USA).

Statistical processing

The data expressed are the mean ± S.D. of triplicate measurement. Analysis of the data was performed statistically using SPSS19.0 software package (IBM Corp., Armonk, NY, USA). The differences were determined between various groups using One-Way Analysis of Variance (ANOVA) followed by Bonferroni's post hoc test. Differences were considered statistically significant at $P<0.05$.

RESULTS AND DISCUSSION

Synthesis of oxymatrine hydrazone

The present study was aimed to synthesize the oxymatrine hydrazone and investigate it against the sevoflurane induced neuron damage. Reaction of oxymatrine with ortho-hydroxy phenyl hydrazine in ethanol as solvent in presence of potassium carbonate as base delivered the required hydrazone in 89% yield (Fig. 2). The purified compound was investigated for treatment of RA.

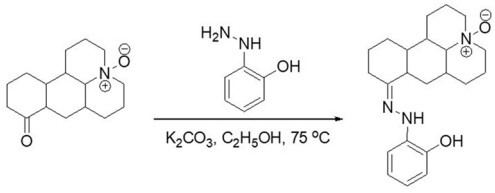

Figure 2. Synthesis of oxymatrine hydrazone.

Effect of oxymatrine hydrazone on viability of neurons

Inhalation of sevoflurane leads to neuro-toxicity in normal people as well as in patients with Alzheimer's disorder (Jang *et al.*, 2017; Lu *et al.*, 2018). In the present study, effect of 0.5, 1.0, 2.0, 4.0 and 5.0 μM doses of oxymatrine hydrazone on viability of neurons was evaluated at 72 h using MTT assay and EVOS Floid Cell imaging Station (Fig. 3). Oxymatrine hydrazone treatment caused no significant change in viabilities of neurons in 0.5–5.0 μM concentration range. The neuronal viabilities at 72 h remained unchanged on exposure to oxymatrine hydrazone in the concentration range of 0.5 to 5.0 μM.

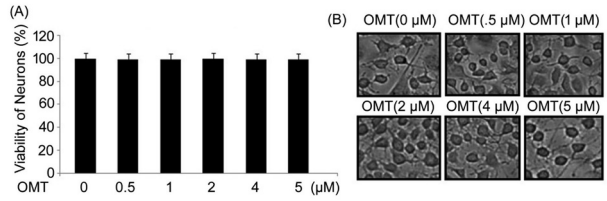

Figure 3. Effect of oxymatrine hydrazone on viability of neurons.
(**A**) Treatment of neurons with 0.5, 1.0, 2.0, 4.0 and 5.0 μM doses of oxymatrine hydrazone was performed for 72 h and viabilities assayed by MTT assay. Values are expressed as the mean ± standard deviation (n=3). (**B**) Examination of oxymatrine treated neurons under EVOS Floid Cell imaging Station.

Oxymatrine hydrazone prevented toxicity of sevoflurane against neurons

Sevoflurane exposure for 6, 12, 24, 48 and 72 h showed toxicity effect on viability of neurons in time-based manner (Fig. 4A). Exposure for 72 h to sevoflurane suppressed viability of neurons to 62% compared to 100% in control. However, oxymatrine hydrazone pretreatment at 0.5, 1.0, 2.0, 4.0 and 5.0 μM doses prevented sevoflurane induced toxicity for neurons in dose-based manner (Fig. 4B). The sevoflurane mediated toxicity for neurons was prevented completely by oxymatrine hydrazone pretreatment at 5.0 μM doses.

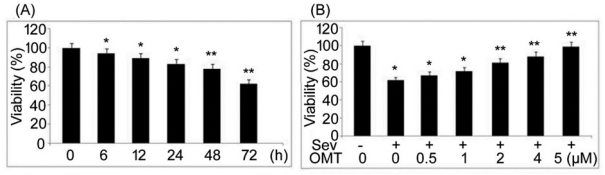

Figure 4. Effect of oxymatrine hydrazone on toxicity induced by sevoflurane.
(**A**) Viabilities of neurons at 6, 12, 24, 48 and 72 h of exposure to sevoflurane were assayed by MTT assay. **P*<0.02 and ***P*<0.01 *vs.* untreated neurons. (**B**) Neurons were pretreated with 0.5, 1.0, 2.0, 4.0 and 5.0 μM doses of oxymatrine hydrazone for 12 h and then exposed to sevoflurane for 72. Values are expressed as the mean ± standard deviation (n=3). **P*<0.05 and ***P*<0.02 *vs.* sevoflurane treated neurons.

Inhibitory effect of oxymatrine hydrazone on apoptosis induced by sevoflurane

In sevoflurane exposed neurons apoptosis was significantly increased compared to control neurons at 72 h (Fig. 5). Apoptotic proportion was increased to 39.08% on exposure to sevoflurane compared to 0.99% in control neurons. However, pre-treatment of neurons with 5.0 μM doses of oxymatrine hydrazone significantly prevented apoptosis induction by sevoflurane.

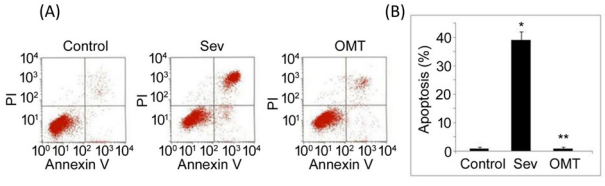

Figure 5. Effect of sevoflurane neuronal apoptosis.
(**A**) The neurons were exposure to sevoflurane for 72 h following oxymatrine hydrazone pre-treatment and then assayed by flow cytometry for apoptosis induction. (**B**) Quantified data from flow cytometry. Values are expressed as the mean ± standard deviation (n=3). **P*<0.05 and ***P*<0.02 *vs.* neurons not exposed to sevoflurane.

Inhibitory effect of oxymatrine hydrazone on sevoflurane induced apoptotic proteins

Exposure of neurons to sevoflurane significantly promoted expression of BAX and cleaved caspases-3 compared to control (Fig. 6). In sevoflurane exposed neurons the expression of BCL-2 was inhibited significantly compared to control. Pre-treatment of neurons with 5.0 μM doses of oxymatrine hydrazone significantly alleviated sevoflurane induced promotion of BAX and cleaved caspases-3. Moreover, sevoflurane mediated reduction of BCL-2 expression in neurons was also prevented by oxymatrine hydrazone treatment. These findings indicate that oxymatrine hydrazone alleviates apoptosis of neurons by suppression of pro-apoptotic protein expression.

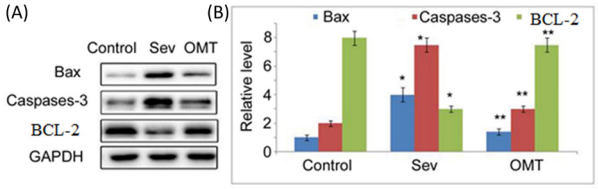

Figure 6. Effect of oxymatrine hydrazone on apoptotic proteins in sevoflurane exposed neurons.
The neurons pre-treated for 24 h with oxymatrine hydrazone were exposed to sevoflurane for 72 h. (**A**) Expression of pro-apoptotic and anti-apoptotic proteins was determined in neurons using western blotting assay. (**B**) Protein levels were quantified. Values are expressed as the mean ± standard deviation (n=3). *P<0.02 and *P<0.01 *vs*. neurons exposed to sevoflurane.

Oxymatrine hydrazone promotes ERK1/2 activation in neurons

Extracellular signal-regulated kinase-1/2 (ERK1/2) has been demonstrated to increase neuronal survival and prevent cell death induced by several insults (Yue *et al*., 2019; Spencer 2003). Moreover, ERK1/2 activation in HT22 cells limits toxicity caused by multiple types of specific insults including serum withdrawal (Colucci-D'Amato *et al*., 2003). Extracellular signals of different nature led to distinct kinetic profiles of activated ERK1/2 and its compartmentalization at different sites within the cell. For example, nerve growth factor mediated ERK1/2 activation in PC12 cells after prolonged exposure leads to its accumulation in nuclei (Spencer *et al*., 2003; Rossler *et al*., 2004). However, rapid activation of ERK1/2 induced by epidermal growth factor fails to bring about its nuclear translocation (Spencer *et al*., 2003; Rossler *et al*., 2004). Studies indicate that kinetics, localization at sub-cellular level and duration of ERK1/2 activation influence downstream targets which subsequently determine promoting or inhibiting effect of ERK1/2 on neuronal survival (Marshall, 1995). Death of neurons, due to inhalation of anesthetic chemicals, is related with the down-regulation of ERK pathway (Chu *et al*., 2004; Anand *et al*., 2011). It is reported that ERK1/2 pathway plays a major role in regulation of neuronal survival following exposure to anesthetic chemicals (Singh & Dhawan, 1997; SÖbbeler *et al*., 2018; Bayes *et al*., 2007; Ma *et al*., 2016, Tanaka *et al*., 1999; Gudbjornsdottir *et al*., 1994; Nicol, 2008; Wang & Zhou, 2018; Osinde *et al*., 2007). In the present study exposure of neurons to sevoflurane caused a marked reduction in phosphorylation of ERK1/2 compared to control (Fig. 7). On the other hand, oxymatrine hydrazone pre-treatment of neurons at 5.0 μM doses prevented sevoflurane mediated suppression of ERK1/2 phosphorylation. The expression of

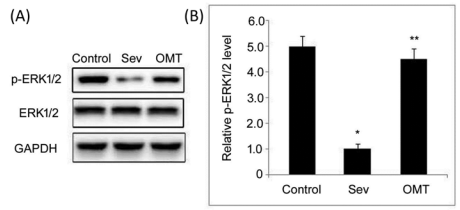

Figure 7. Effect of oxymatrine hydrazone on ERK1/2 phosphorylation.
(**A**) Pretreatment of neurons with 5.0 μM doses of oxymatrine hydrazone without or after sevoflurane exposure was followed by western blotting to measure ERK1/2 phosphorylation. (**B**) The ERK1/2 phosphorylation was quantified. Values are expressed as the mean ± standard deviation (n=3). *P<0.05 and **P<0.02 *vs*. sevoflurane exposed neurons.

phosphorylated-ERK1/2 in control and oxymatrine hydrazone pre-treated neurons was almost similar.

Oxymatrine hydrazone induced ERK1/2 phosphorylation inhibition by MEK inhibitor in sevoflurane exposure neurons

Phosphorylation of ERK1/2 in oxymatrine hydrazone pre-treated and sevoflurane exposed neurons was assayed after U0126 (MEK inhibitor) treatment (Fig. 8). The increased ERK1/2 phosphorylation by oxymatrine hydrazone pre-treatment in sevoflurane exposed neurons was significantly alleviated on treatment with U0126. This indicated that oxymatrine hydrazone prevented sevoflurane induced neurotoxicity through ERK1/2 phosphorylation promotion.

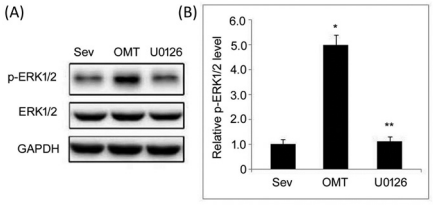

Figure 8. Inhibition of ERK1/2 phosphorylation induced by oxymatrine hydrazone by U0126.
(**A**) Oxymatrine hydrazone pre-treated neurons were treated with U0126, followed by exposure to sevoflurane and assessment of ERK1/2 phosphorylation by western blotting. (**B**) Quantification of ERK1/2 phosphorylation. Values are expressed as the mean ± standard deviation (n=3). *P<0.05 and *P<0.02 *vs*. no neurons not exposed to sevoflurane.

DISCUSSION

Cognitive impairment and gradual memory loss have been reported in people inhaling anesthetic chemicals, such as sevoflurane, due to apoptosis of neurons (Spencer *et al*., 2003; Colucci-D'Amato, 2003). Inhalation of sevoflurane leads to neuro-toxicity in normal people as well as in patients with Alzheimer's disorder (Rossler *et al*., 2004; Marshall, 1995). In the present study, treatment with oxymatrine hydrazone for 72 h could not affect the viabilities of neurons at different concentrations. However, exposure to sevoflurane induced prominent toxicity in neurons which was evident by significant suppression in viabilities with the increase in duration of exposure. Sevoflurane exposure increased apoptosis of neurons significantly at 72 h compared to the control cultures. However, sevoflurane mediated increase in apoptosis of neurons was effectively alleviated on oxymatrine hydrazone pre-treatment. Moreover, in sevoflurane exposed

neurons BAX and cleaved caspases-3 levels were elevated markedly while as level of BCL-2 was inhibited. On the other hand, oxymatrine hydrazone pretreatment reversed elevation of BAX and cleaved caspases-3 and suppression of Bcl-2 in neurons exposed to sevoflurane.

Extracellular signal-regulated kinase-1/2 (ERK1/2) has been demonstrated to increase neuronal survival and prevent cell death induced by several insults (Chu *et al.*, 2004; Anand *et al.*, 2011). Moreover, ERK1/2 activation in HT22 cells limits toxicity caused by multiple types of specific insults including serum withdrawal (Singh & Dhawan, 1997). Extracellular signals of different nature led to distinct kinetic profiles of activated ERK1/2 and its compartmentalization at different sites within the cell. For example, nerve growth factor mediated ERK1/2 activation in PC12 cells after prolonged exposure leads to its accumulation in nuclei (Anand *et al.*, 2011; SÖbbeler *et al.*, 2018). However, rapid activation of ERK1/2 induced by epidermal growth factor fails to bring about its nuclear translocation (Anand *et al.*, 2011; SÖbbeler *et al.*, 2018). Studies indicate that kinetics, localization at subcellular level and duration of ERK1/2 activation influence downstream targets which subsequently determine the promoting or inhibiting effect of ERK1/2 on neuronal survival (Bayes *et al.*, 2007). The death of neurons due to inhalation of anesthetic chemicals is related with the down-regulation of ERK pathway (Ma *et al.*, 2016; Tanaka *et al.*, 1999). It is reported that ERK1/2 pathway plays a major role in the regulation of neuronal survival following exposure to anesthetic chemicals (Gudbjornsdottir *et al.*, 1994; Nicol, 2008; Wang & Zhou, 2018; Osinde *et al.*, 2007; Liu *et al.*, 2015). In the present study, exposure of neurons to sevoflurane caused a prominent reduction in ERK1/2 phosphorylation. However, pretreatment of neurons with oxymatrine hydrazone prevented sevoflurane mediated targeting of ERK1/2 phosphorylation. For confirmation of ERK1/2 activation upregulation in neurons by oxymatrine hydrazone, the neurons were exposed to U0126 (MEK inhibitor) prior to sevoflurane exposure. The data showed that oxymatrine hydrazone pretreatment could not promote ERK1/2 phosphorylation in sevoflurane exposed neurons treated with U0126. This indicated that oxymatrine hydrazone prevented sevoflurane induced neurotoxicity through ERK1/2 phosphorylation promotion.

CONCLUSION

Thus, oxymatrine hydrazone prevents toxicity induction in neurons by sevoflurane through inhibition of cell apoptosis. Oxymatrine hydrazone promoted anti-apoptotic proteins in sevoflurane exposed neurons and inhibited cleaved caspase-3 expression. Additionally, phosphorylation of ERK in sevoflurane exposed neurons was elevated on treatment with oxymatrine hydrazone. Therefore, oxymatrine hydrazone has neuro-protective potential against anesthesia induced toxicity and may be investigated further as a therapeutic agent.

Declarations

Conflict of interest. No conflict of interest is associated with this work

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