Neurotoxicology and Teratology 65 (2018) 19–25

Contents lists available at ScienceDirect

Neurotoxicology and Teratology

journal homepage: www.elsevier.com/locate/neutera

# Emulsified isoflurane induces release of cytochrome C in human neuroblastoma SHSY-5Y cells via JNK (c-Jun N-terminal kinases) signaling pathway

Zeyong Yang[a,*,1], Jingjing Lv[b,1], Xianfu Lu[c], Xingxing Li[c], Xiaohu An[a], Jianwei Wang[a], Hao Weng[d], Yuanhai Li[c,*]

[a] Department of Anesthesiology, International Peace Maternity and Child Health Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200030, PR China
[b] Department of Anesthesiology, The First Affiliated Hospital of Wannan Medical College, No. 2 Zheshan West Road, Wuhu 241000, Anhui, PR China
[c] Department of Anesthesiology, First Affiliated Hospital of AnHui Medical University, No 218 Jixi Road, Hefei 230022, Anhui, PR China
[d] Department of Anesthesiology, Fengxian Central Hospital, Shanghai, Nanfeng Road on the 6600th, Fengxian District, Shanghai 201499, PR China

ARTICLE INFO

*Keywords:*
Emulsified isoflurane
The human neuroblastoma SHSY-5Y cells
JNK signaling pathway
Cytochrome C
Neurotoxicity

ABSTRACT

A large number of studies have demonstrated that inhalation anesthetic isoflurane induced neural cell death by apoptosis in various cell and animal models. Emulsified isoflurane (EIso) is a new type of intravenous preparation of isoflurane that attracts increasing research attention as a promising clinical agent due to its both advantages as an intravenous and inhalation anesthetics medication. However, its safety and underlying molecular mechanism of neurotoxicity largely remain unknown. Therefore, it is meaningful to investigate the safety of EIso and to further elucidate its mechanism of anesthetic neurotoxicity.

Human neuroblastoma SHSY-5Y cells were cultured, followed by a random exposure to one of three doses of EIso (0.56 mmol/l, 1.12 mmol/l, and 2.24 mmol/l) or the corresponding intralipid as vehicle (0.3956 μl/ml, 0.7912 μl/ml and 1.5824 μl/ml) for 6 h, 12 h or 24 h. Cell viability was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide (MTT) assay and the morphological changes were determined by a light microscope. We detect JNK, p-JNK and cytochrome C (cyto C) protein levels by western blotting. SP600125, a specific inhibitor of JNK, was used to detect the role of JNK pathway in the neurotoxicity of EIso.

Our study showed that EIso reduced the viability of SHSY-5Y cells in a dose- and time-dependent manner. 0.56 mmol/l EIso has no significant effects on cell viability, while 1.12 mmol/l of EIso with 24-h and 2.24 mmol/l of EIso with over 12-h exposure notably reduced cell viability. EIso dramatically increased the levels of p-JNK and cyto C. The JNK pathway inhibitor SP600125 significantly increased the cell viability of SHSY-5Y cells induced by EIso.

These findings suggest that EIso induces damage in human neuroblastoma SHSY-5Y cells by JNK signaling pathway activation and cyto C release. SP600125 protects neural cells against EIso-induced injury. Our findings provide a new insight in the exploration of potential novel therapeutic strategies for the treatment of EIso-induced neurotoxicity and other neurodegenerative diseases.

## 1. Introduction

Both preclinical (Culley et al., 2004; Jevtovic et al., 2003) and clinical studies (Monk et al., 2008; Moller et al., 1998: Wilder et al., 2009) demonstrated that sedatives and anesthetic agents administered at the extremes of life may induce long-lasting neurobehavioral changes. Isoflurane is one of the most commonly used volatile anesthetics. However, it may cause severe neuronal apoptosis in both developing animal brains and primary neuronal cells (Noguchi et al., 2017; Schenning et al., 2017; Ge et al., 2015; Brambrink et al., 2010; Kong et al., 2011; Wei et al., 2005).

The JNK pathway is emerging as a central player in neurodegenerative diseases. Its activation is prompted by various neurodegeneration risk factors, including oxidative stress, inflammation, and aging (Lotharius et al., 2005; Pei et al., 2008; Valakh et al., 2015). SP600125 is a potent selective inhibitor of JNK1/2/3, which inhibits the phosphorylation of JNK via reversible competition for ATP and hence suppresses the actions of the JNK signaling pathway. Many studies have

* Corresponding authors.
*E-mail addresses:* yankylge@aliyun.com (Z. Yang), yuanhaili2014@sina.com (Y. Li).
[1] Zeyong Yang and Jingjing Lv contributed equally to this work.

https://doi.org/10.1016/j.ntt.2017.12.001
Received 27 July 2017; Received in revised form 27 November 2017; Accepted 4 December 2017
Available online 06 December 2017
0892-0362/ 

demonstrated that SP600125 protects the central nervous system against ischemia reperfusion injury (Nijboer et al., 2010; Guan et al., 2006a, 2006b). One of the most significant events in apoptosis is mitochondrial dysfunction. The loss of mitochondrial transmembrane potential (MTP) elicits the release of cyto C from the mitochondria to the cytosol, thereby activating the caspase-cascade system (Green and Reed, 1998; Thornberry and Lazebnik, 1998).

A previous study has also shown that activation of caspases promoted the release of cyto C from the mitochondria to the cytosol (Lu et al., 2016). The apoptosome complex formation starts with the release of cyto C, which is a crucial step in the mitochondria-mediated apoptosis. The release of cyto C from the mitochondrial inter-membrane space into the cytosol is a prerequisite of caspase-dependent apoptosis pathway.

Emulsified isoflurane (EIso) is an emulsion formulation of isoflurane with features of both intravenous and inhalation anesthetics. Comparatively, EIso has a smaller minimum alveolar concentration (MAC) value, faster induction, and a lower dosage of application; furthermore, the infused intralipid increases the isoflurane blood/gas partition coefficient (Yang et al., 2006). EIso attracts considerable research interest as a promising clinical agent, while it is still not elucidated whether it induces any neurotoxicity. Therefore, it is meaningful to investigate the safety of EIso and to further examine its mechanism of potential neurotoxicity.

Therefore, we hypothesized that JNK signaling pathway activation contributed to the potential EIso-induced apoptosis with the release of cyto C in a human neuroblastoma SHSY-5Y cell line, which is an attractive system as an in-vitro experimental model of neurodegenerative diseases (Uehara et al., 2006; Meng et al., 2013) for studying pathogenic effects. In the present study, we explored the potential neurotoxicity in SHSY-5Y cells exposed to EIso alone or in combination with a JNK inhibitor, aimed to establish potential beneficial therapies for treatment or attenuation of neurodegenerative disorders.

## 2. Materials and methods

### 2.1. Reagents

Intralipid (30%) was purchased from Sino-Swed Pharmaceutical Corp. Ltd. (Wuxi, China). Emulsified isoflurane (8%) was obtained from the Laboratory of Anesthesiology and Critical Care Medicine, West China Hospital of Sichuan University (Chengdu, China). JNK primary antibody and p-JNK primary antibody were bought from Cell Signaling Technology (Beverly, MA, USA). Cyto C primary antibody, β-actin primary antibody, SP600125 (1,9-pyrazoloanthrone anthrapyrazolone), dimethyl sulfoxide (DMSO), and MTT were purchased from Sigma Aldrich (St. Louis, MO, USA). Fetal bovine serum, Dulbecco's modified Eagle's medium (DMEM), streptomycin, and penicillin G were obtained from Invitrogen (Grand Island, NY, USA).

### 2.2. Cell cultures and treatment

Human neuroblastoma SHSY-5Y cells (kindly provided by Prof. Zunji Ke from the Department of Biochemistry, Shanghai University of Traditional Chinese Medicine, Shanghai, China) were cultured in DMEM containing 10% fetal bovine serum, 100 U/ml of streptomycin and 100 U/ml of penicillin G at 37 °C in a humidified atmosphere with 5% $CO_2$. SHSY-5Y cells were then randomly exposed to three doses of EIso or intralipid for different durations (6 h, 12 h, or 24 h). We chose EIso with final concentrations of 0.56 mmol/l, 1.12 mmol/l, and 2.24 mmol/l to study the EIso-evoked neurotoxicity based on the survival curve of the preliminary experiments and the corresponding intralipid used as a vehicle (0.3956 μl/ml, 0.7912 μl/ml, or 1.5824 μl/ml).

### 2.3. Treatment with a combination of EIso and JNK inhibitor

SHSY-5Y cells that were treated with the JNK inhibitor were randomly divided into six groups: control group with DMSO (supplemented with the same volume of DMSO as that of 10 μM SP600125), control group with SP600125 (supplemented with 10 μM SP600125), 1.12 mmol/l EIso exposure group with the corresponding DMSO or 10 μM SP600125, and the relevant concentration (0.7912 μl/ml) of intralipid group with DMSO or SP600125. The JNK inhibitor SP600125 was added 1 h before SHSY-5Y cells were exposed to EIso. All groups were incubated for 24 h, and then the cellular morphology and cell viability were examined.

### 2.4. MTT assay

Cell viability was tested by the MTT assay as previously described (Yang et al., 2017). The cells, already treated with EIso, were seeded at a density of 15,000 cells/well on 96-well plates. Then, they were incubated for 24 h, and 10 μl of MTT were subsequently added per well, followed with incubation for 4 h in the cell culture incubator, where the samples were placed immediately after the treatment. Further, after 4 h, the medium was aspirated, and 100 μL of DMSO was added in each well to dissolve the purple formazan precipitate, followed by spectrophotometric quantification at 490 nm.

### 2.5. Western blotting

Cells were washed with cold phosphate-buffered saline (PBS, pH 7.4) immediately after the end of the treatment and then lysed with RIPA buffer [150 mM NaCl, 50 mM Tris (pH 8.0), 1% Nonidet P-40 (NP-40), 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholic acid, 0.1 mg/ml phenylmethylsulfonylfluoride, 1 mM sodium orthovanadate, and 10 μg/ml aprotinin mixed with 10 μg/ml leupeptin (a cysteine proteinase inhibitor), 10 μg/ml pepstatin A (an inhibitor of the aspartic protease cathepsin D)] for 30 min on ice. The supernatant containing soluble total protein was collected for analysis of the levels of p-JNK and JNK after centrifugation at 12,000 rpm for 20 min at 4 °C. To detect the cytochrome *c* release, cytosolic protein extracts were prepared. The BCA test was used to evaluate the protein concentrations, and then aliquots the protein samples (40 μg) were loaded into the lanes of a sodium dodecyl sulfate (SDS) polyacrylamide gel. The proteins were separated by electrophoresis at a constant current and pressure, followed by transfer of the separated proteins onto nitrocellulose membranes. The membranes were blocked with 5% non-fat milk powder in 0.01 M PBS (pH 7.4) and 0.05% Tween-20 at room temperature for 1 h. Subsequently, they were incubated with primary antibodies against JNK (1:1000), p-JNK (1:1000), cytochrome C (1:1000), and β-actin (1:2000) overnight at 4 °C by gently shaking, followed by addition of the appropriate horseradish peroxidase-conjugated secondary antibodies (1:3000) and incubation at room temperature. The expression levels of the proteins were quantitatively analyzed through comparison with β-actin as an internal control. The expression was detected by Western Blotting Chemiluminescence (ECL) Detection Kit (Majorbio biological medicine Corp. Ltd., Shanghai, China) after three quick washes in TPBS. Densitometric measurements of the bands were conducted using the Image J software (Schneider et al., 2012).

### 2.6. Statistical analysis

Data were presented as means ± SD. All statistical tests were performed using GraphPad Prism 5.0 (GraphPad Software, Inc., La Jolla, CA). The density of immunoblotting was determined by the software of Image J. Measurement data were compared by two-ways ANOVA analyses, and Bonferroni's multiple comparison tests were conducted for all samples. Statistical significance was set at a level of $P < 0.05$.