Original Research Article

CS nerve growth factor regulates sevoflurane anesthesia-induced nerve injury in nerve cells

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Sent for review: 22 Mar 2020 Revised accepted: 26 April 2020

Abstract

Purpose: Sevoflurane is the most commonly used anesthetic agent for surgery. However, it is associated with deficiency in learning and memory abilities. The study was aimed at investigating the role of nerve growth factor (NGF) in sevoflurane anesthesia-induced nerve injury.

Methods: RT-qPCR assay was applied to measure expressions of NGF, miR-98-5p and other factors related to apoptosis. CCK-8 assay was used for detecting cell viability while luciferase reporter assay was employed to measure binding condition between miR-98-5p and NGF. Expressions of proteins in PI3K/AKT/mTOR signaling pathway was measured with western blot.

Results: Sevoflurane reduced cell viability of RGC-5 cells, promoted apoptosis and reduced the expression of NGF. In sevoflurane-induced RGC-5 cells, over-expression of NGF promoted cell viability with reduced apoptosis. Also, there was reduction in the protein expression of PI3K/AKT/mTOR signaling pathway by sevoflurane, while up-regulation of NGF promoted the expressions of these proteins. In the presence of PI3K inhibitor, reduction cell viability was reduced but apoptosis increased. Luciferase reporter assay detected MiR-98-5p as the target gene of NGF and its overexpression restored high cell viability in the over-expressed NGF. The rate of apoptosis and expressions of proteins was also restored with up-regulation of miR-98-5p.

Conclusion: Sevoflurane caused damage to nerve cells, while over-expression of NGF reduced the injury through PI3K/AKT/mTOR signaling pathway and suppression of miR-98-5p.

Keywords: Nerve growth factor, Sevoflurane, Nerve injury, Anesthesia, miR-98-5p

INTRODUCTION

Generally, anesthesia is commonly used for surgery. It is a reversible, drug-induced condition associated with loss of consciousness, amnesia, analgesia and dyskinesia. Meanwhile, the patient still has the stability of the autonomous nervous, cardiovascular, respiratory and thermoregulatory systems [1]. For research and diagnostic purposes, surgery is also a kind of wound, while successful implementation of anesthesia helps surgery become better and develops rapidly as a new way to treat diseases. Sevoflurane was first discovered by Bernard M. Regan in 1968 and its pharmacological action, physicochemical and toxicological properties were reported in 1975 by Willin et al. After that, it was adopted for clinical application in 1990 in Japan as anesthetic agent.
The two ways in which sevoflurane inhalation are used for clinical application include lung volume breathing and tidal volume breathing induction. Nowadays, sevoflurane is the most common inhaled anesthetics with stable physical and chemical properties. It has easy acceptable smell and no irritation to respiratory tract. Thus, sevoflurane can be a perfect induced medicine in pediatric anesthesia. Moreover, sevoflurane has hemodynamic stability and rarely causes bradycardia. Also, it has no impact on abilities of liver and kidney with muscular flaccidity and quick awakening, which make it suitable for low-flow anesthesia [2,3]. Besides that, some side effects of sevoflurane were detected as well, which indicated that brain development could be damaged with its use as anesthetic agent. Also, researches have proven that juvenile rats exposed to sevoflurane could acquire nerve cell apoptosis, as well as impaired spatial memory [4-7]. Therefore, it is essential for researchers to investigate the mechanism of action of sevoflurane and to find out methods for reducing its damages to nervous system.

Nerve growth factor (NGF) belongs to neurotrophic factor family, which can accelerate differentiation and maintain survival of neurons. NGF was first discovered by Rita Levi-Montalcini and Stanley Cohen in 1956, which is an important milestone for modern biological development. As a result of the discovery, both of scientists were awarded Nobel prizes for Medical Physiology in 1986 [8]. NGF was first isolated from snake venom and later from the mouse submandibular gland, especially in male mice [9,10]. Several researches have shown that NGF of mouse has almost 90% homology to that of human and it is convenient to extract NGF from mice, which made it became the first listed NGF for clinical usage [11]. In the bodies of mammals, there are 4 kinds of neurotrophic factors: NGF, BDNF, NT-3 and NT-4/5, which can bind to four kinds of receptors: p75NTR, TrkA, TrkB and TrkC, to play their roles. All of these could bind to p75NTR, but they bind selectively to the Trk receptors [12]. NGF could bind to two kinds of receptors: TrkA and p75NTR in which TrkA is a high affinity NGF receptor and p75NTR is the lower one. TrkA could promote nerve cells proliferation and survival through PI3K-AKT and Ras-MAP signaling pathways, while p75NTR could help in the apoptosis of nerve cells. In order to investigate the functions of NGF in anesthesia-induced nerve injury, correlation between NGF and sevoflurane was analyzed in this study.

The purpose of this study was to investigate the role of nerve growth factor (NGF) in sevoflurane anesthesia-induced nerve injury.

**EXPERIMENTAL**

**Cell culture**

Nerve cell line of mice RGC-5 was purchased from ATCC (USA). The cells were incubated at 37℃, 5% CO₂ in Dulbecco’s Modified Eagle’s Medium (DMEM) (Thermo Fisher, USA) with 10% Fetal Bovine Serum (FBS) (Thermo Fisher, USA). After incubation, cells were mixed with sevoflurane and further cultured for another 24h.

**Cell transfection**

After the nerve cells were cultured, cells in log phase were selected and the mammalian expression vector, pcDNA3.1 was used to overexpress NGF, according to the manufacturer’s instruction. Lipofectamine™ 3000 Transfection Reagent (Thermo Fisher, USA) was used as the reagent of transfection. Before transfection, Opti-MEM medium was used to replace DMEM medium with Lipofectamine™ 3000 added. After the cells were transfected overnight, cells were moved to DMEM medium for 48h incubation. Thereafter, expressions of NGF and miRNA were detected through RT-qPCR.

**RT-qPCR for measurement of gene expression**

Total cellular RNAs were extracted from transfected cells with Trizol reagent (Thermo Fisher, USA) following the manufacturer’s instruction. The isolated RNAs were reverse transcribed to cDNAs using High Capacity cDNA Reverse Transcription Kit (Thermo Fisher, USA). Several oligonucleotide primers were used for RT-qPCR amplification. The nucleotide sequences of the primers used are shown in Table 1. The RT-qPCR conditions include: pre-denaturation step of 95℃ for 5min; followed by 35 cycles of denaturation step of 95℃ for 30s; annealing step of 65℃ for 30s and extension step of 72℃ for 30s. Expressions of RNAs were calculated using 2^ΔΔCt method.

**CCK-8 assay for detection of cell viability**

Cells were seeded in 96-well plate with 5×10³ cells per well, after which the cells were incubated at 37℃, 5% CO₂. After cell confluences reached 90%, 10μl CCK-8 was added into each well and was incubated for 2h. This was followed by measurement of optical density (OD) value of cells with microplate reader (Thermo Fisher, USA).
Table 1: Primer sequences used for RT-qPCR

| Target  | Direction | Primer sequence                       | Reference |
|---------|-----------|---------------------------------------|-----------|
| NGF     | Forward   | 5'-ACACTGAGGTGCATAGCGTA-3'            | 13        |
|         | Reverse   | 5'-GTTTAGTCCAGTGGGCTTGG-3'            |           |
| Bcl-2   | Forward   | 5'-CGCCCGCTGTGCACCGAGA-3'             |           |
|         | Reverse   | 5'-CATGTGTGTGAGAGCGGCTTGG-3'          |           |
| p53     | Forward   | 5'-CCAGGGCAAGCTACGGTTTC-3'            |           |
|         | Reverse   | 5'-CATGTGTGTGAGAGCGGCTTGG-3'          | 14        |
| Caspase-3| Forward | 5'-TGAGGTAGTAGTTTGTGCTGTT-3'          | 15        |
| GAPDH   | Forward   | 5'-CTCGCTTCGGCAGACACA-3'              |           |
| miR-98-5p| Reverse  | 5'-CACGACGACAAGATTTACGAC-3'           |           |

Luciferase reporter assay for evaluation of binding condition between miR-98-5p and NGF

Using bioinformatics tools, NGF sequence was predicted to combine with miRNA. Wild type and mutant sequences of NGF were synthesized and cloned into the pmirGLO Dual-Luciferase miRNA target expression vector (Kalang, Shanghai, China) to construct pmirGLO-NGF-wt and pmirGLO-NGF-mut vectors. In the luciferase reporter assay experiment, miRNA mimics (Thermo Scientific, USA), miRNA inhibitor with pmirGLO-NGF-wt, pmirGLO-NGF-mut, Negative inhibitor and Negative mimics were co-transfected into RGC-5 cells using Lipo293TM Transfection Reagent (Beyotime, Shanghai, China). After transfection for 24h, cells were collected and analyzed through the Dual-Luciferase Reporter assay system (Thermo Fisher, USA) following the manufacturer’s instructions.

Western blot for measurement of protein level

Cells were lysed by RIPA reagent (Beyotime, Shanghai, China) to extract total proteins and the proteins were quantified using BCA method (Beyotime, USA) and transferred onto PVDF membranes (Beyotime, Shanghai, China). The membranes were blocked with 10% skimmed milk powder for 4h and incubated with anti-PI3K (1:1000; ab, Cambridge, UK), anti-AKT, anti-mTOR and GAPDH (1:2000; ab181602) overnight at 4°C. Then, secondary antibodies, Goat Anti-Rabbit IgG (1:800; ab150077) and Goat Anti-Mouse IgG (1:800; ab150117) marked with HRP were added and incubated with membranes at room temperature for 2h. Specific proteins were measured through Click-IT™ Biotin Protein Analysis Detection Kit (Thermo Fisher, USA). GAPDH was treated as the standard.

Statistical analysis

Each experiment was replicated three times and all data were presented as mean±SD. Data were analyzed using SPSS 19.0 (IBM, USA). P<0.05 was considered to have statistical significance.

RESULTS

Sevoflurane induced apoptosis of nerve cells and suppressed expression of NGF

RGC-5 cell line was selected for measuring the functions of sevoflurane. After RGC-5 cells were exposed to sevoflurane, cell viabilities of normal RGC-5 and induced RGC-5 were detected. Compared to normal cells, induced RGC-5 showed less viabilities of cells (Figure 1A). Furthermore, factors of apoptosis in RNA level was detected, which showed that in induced RGC-5, Bcl-2 expression was reduced (Figure 1B), while p53 (Figure 1C) and caspase-3 expressions (Figure 1D) were increased. The expression level of NGF in normal RGC-5 and induced group was measured and the results obtained indicated that expression was higher in normal RGC-5 than induced group (Figure 1E).

Up-regulation of NGF alleviated nerve injury caused by sevoflurane

After the expression of NGF was measured in normal and induced cells, transfected NGF expression was analyzed in induced RGC-5. When the results obtained were compared with negative control group, overexpressed NGF increased the level of NGF (Figure 2A). Moreover, up-regulated NGF increased cell viabilities in induced RGC-5 (Figure 2B). Apoptosis of cells was measured and the results
Figure 1: Sevoflurane caused apoptosis of nerve cells and inhibited expressions of NGF. A: Cell viabilities were detected through CCK-8, P<0.05; B, C, D: RT-qPCR was applied to measure expressions of Bcl-2, p53 and caspase-3 respectively, P<0.05; E: Expressions of NGF were evaluated by RT-qPCR, P<0.05.

Figure 2: Decrease in sevoflurane-induced nerve injury due to overexpression of NGF. A: Overexpression of NGF was measured through RT-qPCR, P<0.05; B: CCK-8 assay was used for detecting viabilities of RGC-5 cells in negative control (NC) and overexpressed NGF groups, P<0.05; C, D, E: Bcl-2, p53 and caspase-3 expressions were validated using RT-qPCR, P<0.05.
obtained showed that Bcl-2 expression was higher in overexpressed NGF (Figure 2C), while there was a decrease in the level of expressions of p53 (Figure 2D) and caspase-3 (Figure 2E) genes.

NGF regulated sevoflurane anesthesia-induced nerve injury through PI3K/AKT/mTOR signaling pathway

After the functions of NGF were evaluated, related signaling pathway was investigated. In normal and induced cells, expressions of proteins in PI3K/AKT/mTOR signaling pathway were validated through Western blot. There was a decrease in the protein expression levels of PI3K/AKT/mTOR in induced RGC-5 (Figure 3A). The expressions of proteins in PI3K/AKT/mTOR signaling pathway were measured in the overexpressed NGF. The results obtained (Figure 3B) indicated that overexpression of NGF led to the up-regulation of PI3K, AKT and mTOR. In order to confirm that PI3K/AKT/mTOR signaling pathway was involved, cell viabilities were measured after protein in the signaling pathway was inhibited. As LY294002 was applied, higher cell viabilities were observed when NGF was overexpressed (Figure 3C). Meanwhile, expression of Bcl-2 was also inhibited with increasing expressions of p53 and caspase-3 (Figure 3D).

MiR-98-5p was the target of NGF with regulating nerve injury induced by sevoflurane

With the aid of Target Scan, miR-98-5p was predicted to have binding site with NGF. In order to confirm the binding situation, luciferase reporter assay was applied to determine whether NGF would bind to miR-98-5p or not. The results obtained (Figure 4A) suggested that miR-98-5p wild type was the target of NGF and not the mutant type. The correlation between miR-98-5p and NGF was determined. In induced RGC-5 cells, up-regulated miR-98-5p had increased expression by overexpressed NGF (Figures 4B and C). Moreover, the increased cell viabilities were also suppressed with addition of overexpressed miR-98-5p (Figure 4D). Apoptosis was detected as well, which indicated that Bcl-2 expression decreased after overexpressed miR-98-5p was added, while p53 and caspase-3 expressions were significantly increased (Figure 4E). Furthermore, expressions of proteins after the addition of up-regulated miR-98-5p were measured, which showed that high expressions of PI3K, AKxwT and mTOR with overexpressed NGF was reversed by overexpression of miR-98-3p (Figure 4F).

Figure 3: NGF regulated sevoflurane induced nerve injury via PI3K/AKT/mTOR signaling pathway. A: Expressions of proteins in normal RGC-5 cells and sevoflurane treated RGC-5 cells were measured by western blot; B: Expressions of proteins in NC group and overexpressed NGF group were measured by western blot; C: CCK-8 was applied to detect cell viabilities, P<0.05; D: Bcl-2, p53 and caspase-3 expressions were validated using RT-qPCR, P<0.05.
Figure 4: MiR-98-5p was the target of NGF with regulating nerve injury induced by sevoflurane. A: Target Scan was used for predicting binding site between NGF and miR098-5p; B: Luciferase reporter assay was used for evaluating binding conditions between NGF and miR-98-5p, $P<0.05$; C: Relative expressions of NGF with overexpressed miR-98-5p were detected through RT-1PCR, $P<0.05$; D: Cell viabilities after the addition of miR-98-5p mimics were validated through CCK-8 assay, $P<0.05$; E: Bcl-2, p53 and caspase-3 expressions were evaluated via RT-qPCR, $P<0.05$; F: Levels of proteins were detected via Western blot

DISCUSSION

In China, there are several patients receiving anesthesia in clinical treatment every year. Thus, it is worthy to investigate whether the anesthetics could cause neurovirulence or adverse impacts to patients' memory function. Sevoflurane is the most common inhalation anesthetic agent used in surgery, but data showed that sevoflurane could cause cognition impairment. However, the mechanism by which this occurs still need to be confirmed [16]. Related researches have suggested that when a pregnant mice inhaled sevoflurane at a concentration of 2.5% for 2h and after birth, led to decline in spatial memories of offspring mice [17]. Another study indicated that there was no decrease in spatial memories of offspring mice given birth to from a pregnant mice, when sevoflurane was inhaled at a concentration of 1.5% for 6h [18]. Moreover, from previous studies, sevoflurane was proven to cause apoptosis of nerve cells, leading to decline in learning and memory abilities, especially its pro-apoptosis ability to developing nerve cells [19]. Researches have also elucidated the pathway of apoptosis by sevoflurane, which indicated that sevoflurane could increase level of caspase-3 and mitochondrial pathway to take part in apoptosis [20]. Besides that, sevoflurane could also activate FASL-FAS signaling to promote apoptosis of nerve cells [21]. Other researches have also proven that sevoflurane could help phosphorylation of PERK, which is a marker of apoptosis through endoplasmic reticulum, activating a series of signal molecules in down-stream to promote apoptosis [22]. Therefore, this study was also carried out to investigate the functions of sevoflurane in nerve injury. By comparing RGC-5 in normal condition with those exposed to sevoflurane, cell viabilities in exposed group were significantly lower than those in normal condition, which suggested that sevoflurane could reduce proliferation of nerve cells. Furthermore, in order to determine its effect on apoptosis, factors were evaluated as well. The results obtained from the study indicated that there was a reduced expression level of Bcl-2 in induced RGC-5 when compared with normal RGC-5, while caspase-3 and p53 expressions were significantly increased. Thus, sevoflurane was suggested to cause damage of nerve cells and suppressed proliferation by promoting apoptosis.

NGF is a specific protein molecule, which plays an essential role in growth, survival, differentiation, self-protection and repair of nerve damage. NGF is mainly located in nerve target
cells, such as neurogliocytes, skeletal muscles, schwann cells and so on. There are some reports suggesting that NGF could be extracted from other tissues and organs like serum, saliva and urine [23]. NGF extracted from mice submaxillary gland could effectively cure peripheral nerve injury, keratohelcosis and glaucoma, which in turn could also be used to treat radioactive temporal lobe necrosis caused by radiotherapy for nasopharyngeal cancer [24-26]. Therefore, NGF expression was analyzed in RGC-5 cells, which showed that sevoflurane could reduce the level of NGF. After NGF was overexpressed, cell viabilities in induced RGC-5 cells were increased as well. Moreover, Bcl-2 expression was up-regulated and the expressions of p53 and caspase-3 were decreased. The results obtained from this study showed that NGF was the factor that protects nerve cells' abilities.

PI3K/AKT was the most important signaling pathway related to survival of nerve cells, which caused attentions in metabolism, proliferation and apoptosis. Moreover, the pathway is widely present in nervous system and takes part in survival, differentiation and apoptosis of gliocyte and nerve cells [27, 28]. Researches have proven that many diseases had connections with confusion of PI3K/AKT signaling pathway, among which include cancer, degenerative diseases of the nervous system and diabetes mellitus. PI3K/AKT signaling pathway also has connections with changes of nerve and pathological structure in Alzheimer's disease [29]. Therefore, in this research, the functions of PI3K/AKT/mTOR signaling pathway was measured. In induced RGC-5 cells, expressions of proteins in PI3K/AKT/mTOR signaling pathway were also lower than those in normal cells. After the expression of NGF was increased, up-regulations of those proteins were observed. To confirm the function of PI3K/AKT/mTOR signaling pathway, PI3K suppressor was applied, which reduced the high expressions of overexpressed NGF and helped in apoptosis after the inhibitor was added. Moreover, microRNAs could bind mRNAs to inhibit their expressions. In this study, miR-98-5p was the target gene that bound to NGF. As mimics of miR-98-5p was added, increase in cell viabilities by NGF was reversed, as well as apoptosis. Moreover, protein expressions were also restored with overexpression of miR-98-5p. Thus, PI3K/AKT/mTOR was the signaling pathway which was involved in the nerve injury by sevoflurane.

**CONCLUSION**

The findings from this research indicated that sevoflurane caused nerve injury and NGF was the factor capable of repairing the damage caused through PI3K/AKT/mTOR signaling pathway. Also, miR-98-5p contributed to the process by binding to NGF in nerve cells. Further research is required to fully elucidate the steps involved in the mechanism of action.

**DECLARATIONS**

**Acknowledgement**

None declared

**Conflict of interest**

No conflict of interest is associated with this work.

**Contribution of authors**

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. All authors read and approved the manuscript for publication.

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