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Sevoflurane affects neurogenesis through cell cycle arrest via inhibiting wnt/β-catenin signaling pathway in mouse neural stem cells

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ABSTRACT

Aims: The development of central nervous system requires proliferation of neural stem cells followed by differentiation. Cell cycle parameters are closely related with cell fate specification and differentiation. Recent researches indicated that wnt/β-catenin signaling pathway might cause proliferation inhibition and differentiation abnormality through interfering NSCs cell cycle. Our previous research also showed that multiple sevoflurane exposure to neural stem cells inhibited proliferation via repressing transcription factor Pax6 and cyclin D1 through inhibiting wnt/β-catenin pathway. All above encouraged us to figure out the effect of sevoflurane on cell cycle and neurogenesis.

Main methods: Primary mouse cultured neural stem cells were used and exposed to 4.1% sevoflurane for 6 h in this study. The expression of β-catenin, GSK-3β, c-myc and cyclin D1 were determined by western blot and qRT-PCR. FACS was used to measure the cell cycle. The proliferation of NSCs was evaluated by EdU staining while the differentiation was evaluated by Tuj1 and GFAP staining on immunocytochemistry.

Key findings: We found that exposure to sevoflurane at a concentration of 4.1% for 6 h induced inhibition of wnt/β-catenin pathway, cell cycle arrest at G0/G1 phase and an earlier switch from proliferation to differentiation. GSK-3β specific inhibitor, CHIR99021, attenuated sevoflurane-induced cell cycle arrest and abnormality of neurogenesis in neural stem cells.

Significance: Our research suggested that sevoflurane arrested cell cycle at G0/G1 phase through inhibition of wnt/β-catenin signaling pathway thus resulting in a premature differentiation in NSCs. This study presents a deeper understanding of the mechanism on cognitive impairment by sevoflurane exposure.

1. Introduction

Recent population researches indicated that children who received surgery under general anesthesia in infancy may cause subtle changes in neurodevelopmental outcomes [1–5]. Further research suggested that single, relatively brief exposure to surgical procedures under general anesthesia are not associated with detectable deficits in most cognitive domains or academic achievement while multiple exposure to anesthesia may cause modest neurodevelopmental deficits [6]. In accordance with clinical results, animal studies showed that only multiple exposure to sevoflurane in both new born and prenatal mice induced cognitive impairment [7–9]. The main neurotoxicity mechanism of sevoflurane included apoptosis of neuron, proliferation inhibition and differentiation abnormality in neural stem cells [10–12].

Zhang et al. showed that sevoflurane inhibited proliferation and the wnt/β-catenin signaling pathway in mouse neural progenitor cells [13]. Since the transcriptional control of G0/G1 cell cycle regulators by β-catenin, such as c-myc and cyclin D1, is important for the neural stem cell to make the decision between self-renew and differentiation, we focused on how sevoflurane affects cell cycle and neurogenesis by regulating wnt/β-catenin pathway [14].

In accordance with Zhang's study, our previous study showed that multiple sevoflurane exposure to pregnant mice caused the decrease of Pax6 and cyclin D1 expression which caused the inhibition of NSCs proliferation. Lithium, an inhibitor of wnt/β-catenin/Pax6 pathway, could mitigate this effect [15]. However, only G1 phase was examined by cyclin D1 application, while S and G2 phase remained unknown. A more comprehensive method such as FACS should be used to get a whole picture of cell cycle. Meanwhile, we used lithium, a non-specific GSK-3β inhibitor, to test our hypothesis. A specific GSK-3β inhibitor should be used for further study. Moreover, this study only measured the effect of sevoflurane on proliferation. Since few researches

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evaluated how sevoflurane affected differentiation in NSCs, we paid more attention to the effect of sevoflurane on differentiation.

Our resent paper has already showed that the sevoflurane neurotoxicity may be due to the abnormality of cell cycle which consequently led to proliferation inhibition and differentiation abnormality in the fetal PFC [16]. All together gives us a new insight into how sevoflurane affects proliferation and differentiation through cell cycle changing.

Here, we designed an in vitro study to test the hypothesize that sevoflurane may induce neurogenesis abnormality through interfering cell cycle via inhibition of wnt/β-catenin signaling pathway in mouse neural stem cells.

2. Materials and methods

2.1. Cell culture

Mouse NSCs were isolated from hippocampus of C57BL/6 mouse on day 13.5 of gestation (E13.5) and cultured by a neurosphere method as described previously [17]. The isolated cells were plated in serum-free medium of DMEM/F12 (Gibco) supplemented with 2% B27 (Gibco) without vitamin A, 5 mM L-Glutamine (Gibco), 100 U/ml penicillin and streptomycin (Gibco), 20 ng/ml epidermal growth factor (EGF, Gibco) and 20 ng/ml basic fibroblast growth factor (bFGF, Gibco). The culture medium was replaced every other day. Cells were incubated for 5 days to form enough neurospheres which were then passed at a cell density of 2×10^5/ml.

2.2. Anesthetic exposure

NSCs were exposed to a gas mixture in a gas-tight chamber placed in the incubator at 37 °C, and the concentration of sevoflurane was precisely controlled through a sevoflurane-specific vaporizer (Abbott Laboratories). CHIR99021 (Selleck Inc) is a specific inhibitor of GSK-3β [18]. The cells were randomly assigned into four groups as CON (no inhibitor added, no exposure to sevoflurane), CON + I (inhibitor added, no exposure to sevoflurane), SEV (no inhibitor added, exposed to sevoflurane) and SEV + I (inhibitor added, exposed to sevoflurane).10 nM CHIR99021 was added to NSCs in CON + I and SEV + I group 30 min before exposure. NSCs in SEV and SEV + I group were exposed to a gas mixture which contains 4.1% sevoflurane, 5%CO_2 and 95% air for 6 h. After exposure, the cells were taken out to proceed with the corresponding assays.

2.3. Immunocytochemistry

Since NSCs can automatically differentiate into neurons, oligodendrocytes, and astrocytes, the NSCs specific maker nestin was used to determinate the proportion of NSCs. After exposure to sevoflurane, NSCs were seeded on poly-L-lysine (PLL) pre-coated glass coverslips for 2 h. The cells were mixed with 4% paraformaldehyde for 30 min and washed by PBS three times. After blocked in 0.3% Triton X-100 and 10% fetal bovine serum (FBS, Gibco) in PBS for 1 h at room temperature, the cells were incubated with primary antibody nestin (1:200, Abcam) overnight at 4 °C. The cells were then washed three times with PBS, followed by incubation with secondary antibody (1:200, Alexa FluorR 488) for 1 h at room temperature. After three-time washes, the cells were incubated with 1× DAPI for 3 min at room temperature followed by washing with PBS three times. The glass coverslips were then mounted with mounting medium and imaged.

2.4. Western blot

The protein expression of β-catenin, GSK-3β, c-myc and cyclin D1 was measured by western blot analysis. Cells were centrifuged at 1000 rpm for 5 min at 4 °C and then solubilized with lysis buffer containing protease supplemented with 1 mM PMSF. The cell lysates were centrifuged at 14000 rpm for 15 min at 4 °C. The total protein lysates were separated by SDS-PAGE and were transferred to nitrocellulose membranes. The membranes were incubated with blocking buffer for 2 h at room temperature and primary antibody containing β-catenin (1:1000, cell signaling technology), GSK-3β (1:1000, cell signaling technology), c-myc (1:1000, cell signaling technology), cyclin D1 (1:1000, cell signaling technology), GADPH (1:2000, Santa Cruz) and β-actin (1:2000, Santa Cruz) at 4 °C overnight. After washed by TBST three times, the membranes were incubated with goat anti-rabbit IgG (1:2000, LI-COR) and goat anti-mouse IgG (1:2000, LI-COR) for 45 min at room temperature, followed by washing with TBST three times. The staining was quantified by scanning the films, and the band density was determined with Image-J software.

2.5. qRT-PCR

Quantitative Real-time PCR was performed to evaluate the mRNA expression level of β-catenin, GSK-3β, c-myc and cyclin D1. For RNA isolation, the centrifuged cells were lysed with cell lysis buffer (EZBioscience) for 5 min. The cell lysate was then added to RT Enzyme (EZBiocsience) and RT buffer (EZBioscience) for 25 min at 42 °C for reverse transcription. With the obtained cDNA as a temple, it was added to SYBR Green qRCR Master Mix (EZBioscience), forward primer (BioTNT), reverse primer (BioTNT) and ROX Reference Dye (EZBioscience) for PCR program. All data for each sample were measured in triplicate and using $2^{-\Delta\Delta Ct}$ method.

The sequences of the primers for RT-PCR are as follows,
β-catenin: Forward, 5′-TCA TCT GAC CAG CCG ACA TC-3′.
β-catenin: Reverse, 5′-CAC CCT GTT CCC GCA AAG-3′.
GSK 3β: Forward, 5′-TGG GTC ATT TGG TGT GGT AT-3′.
GSK-3β: Reverse, 5′-GCA ATC GGA CTA TGT TAC AG-3′.
c-myc: Forward, 5′-GTG AGA GTA AGG AGA ACG GT-3′.
c-myc: Reverse, 5′-GCC AAG GTT GTG AGG TTA G-3′.
cyclin D1: Forward, 5′-CAA GGA GAT TGG GGA CAA C-3′.
cyclin D1: Reverse, 5′-TTG CTT TGA GTC ACA CTG GT-3′.
β-actin: Fordward, 5′-CCT CTA TGC CAA CAC AGT-3′.
β-actin: Reverse, 5′-AGC CAC CAA TCC ACA CAG-3′.

2.6. Cell cycle analysis by FACS

Cells were collected by low speed centrifugation (1000 rpm for 5 min) and washed twice with PBS. After fixed with cold 70% ethanol overnight at −20 °C, the cells were suspended in RNase A (Keygentec Inc) for 30 min at 37 °C and propidium iodide (Keygentec Inc) for 30 min at 4 °C. Then they were subjected to cell cycle analysis using FACS and ModFitLT Software.

2.7. Cell proliferation assay

We used EdU staining to measure the cell proliferation activity of NSCs. EdU was added into culture medium 5 min before CHIR99021 was added. After 6 h of incubation, cell proliferation was determined by EdU cell proliferation assay kit. The cells were pretreated with 4% paraformaldehyde for 30 min and 0.5% Titron X-100 for 15 min. Then the prepared the Click-iT reaction cocktail (Keygentec Inc) was applied to the cells for 30 min. The cell nuclei were stained by incubating sections in Hoechst 33342 (Keygentec Inc) solution for 15 min. The glass coverslips were then mounted with mounting medium and imaged. At least 10 fields were selected randomly in each slice and the percentage of EdU^+/$Hoechst^+$ NSCs in $Hoechst^+$ NSCs was calculated.

2.8. Cell differentiation assay

Cell differentiation activity was determined by immunocytochemistry on Tuj1 and GFAP which respectively represented newborn neuron and glial cells. After exposure to sevoflurane, the cells