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Dexmedetomidine-mediated neuroprotection against sevoflurane-induced brain development abnormality in fetal mice brain

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Abstract. – OBJECTIVE: Brain development is susceptible to external influences during the gestation period so the neurotoxicity of anesthetics has gained a lot of attention. We aimed to investigate the neurotoxicity of sevoflurane to fetal mice brain as well as the neuroprotective effects of dexmedetomidine.

MATERIALS AND METHODS: Pregnant mice were treated with 2.5% sevoflurane for 6 hours. The changes in fetal brain development were assayed with immunofluorescence and western blot. The pregnant mice were intraperitoneally injected with dexmedetomidine or vehicle from gestation day (G) 12.5 to G15.5.

RESULTS: Our results showed maternal sevoflurane exposure could not only inhibit neurogenesis but also lead to precocious generation of astrocytes in fetal mice brains. The fetal mice brain of sevoflurane group exhibited a significant inhibition in the activity of Wnt signaling and the expression of CyclinD1, Ngn2. Chronic dexmedetomidine administration could minimize the negative effects caused by sevoflurane by activating the Wnt signaling pathway.

CONCLUSIONS: This study has uncovered a Wnt signaling-related mechanism of the neurotoxicity of sevoflurane and confirmed the neuroprotective effect of dexmedetomidine, which could provide pre-clinical evidence for clinical decision-making.

Key Words:
Sevoflurane, Pregnant, Fetus, Neurotoxicity, Dexmedetomidine, Neurogenesis.

Introduction

Some scholars[1] have associated maternal anesthesia exposure with neurobehavioral abnormalities in offspring, such as attention deficit and cognitive dysfunction. Sevoflurane, one of the commonly used inhaled anesthetics, is thought to have the least neurotoxicity to brain development when compared to other inhalational anesthetics, such as isoflurane or desflurane[2]. However, sevoflurane could permeate through the placental barrier and easily affect the developing fetal brain. Many animal studies[3,4] proved the harmful effect of sevoflurane to the developing brain, such as abnormal neurogenesis, apoptosis and inflammatory response. The proliferation and differentiation are very active in fetal brain and sensitive to drug and environmental stimulation. We have reported a significant decrease in fetal prefrontal cortex neurogenesis after maternal sevoflurane exposure in mid-gestation[5].

Most surgeries during pregnancy are complicated and anesthetic exposure is unavoidable. Therefore, appropriate intervention is essential to decrease the neurotoxicity of anesthetics to fetal brain development. Dexmedetomidine is a highly selective α2-adrenergic receptor agonist in central nervous system. Former studies[2] have reported pre-administration of dexmedetomidine could prevent neonatal mice from sevoflurane-induced apoptosis. The number of the studies focused on the neuroprotective effect of dexmedetomidine is still very limited. A direct link between improvements in cognitive functions and dexmedetomidine consumption still needs to be established.

In the current study, we studied the fetal brain changes with assessing the specific neural marker related to proliferation and differentiation. The transformation of cell cycle of neural stem cells was also investigated with BrdU incorporation and immunochemical analysis of specific cell cycle markers. We also explored the role of Wnt signaling pathway in the sevoflurane-related neurotoxicity and investigated the neuroprotective effect of dexmedetomidine.

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Materials and Methods

Mice Anesthetic Exposure and Dexmedetomidine Treatment

All procedures were approved by the Animal Care and Use Committee of Qingdao University. Three- to four-month-old C57BL/6J female mice were mated with same age male mice. The pregnant mice were raised separately and were randomly assigned to four groups at G15.5 as Con (no dexmedetomidine added, no sevoflurane exposure, n=5, weight 80-100 g), Dex (dexmedetomidine added, no sevoflurane exposure, n=5, weight 80-100 g), Sevo (no dexmedetomidine added, sevoflurane exposure, n=5, weight 80-100 g) and Sevo + Dex (dexmedetomidine added, sevoflurane exposure, n=5, weight 80-100 g). The mice in Con and Sevo were treated with intraperitoneal injection of physiological saline from G12.5-G15.5, while the mice in Dex and Sevo+Dex were treated with dexmedetomidine (50 μg/Kg) from G12.5-G15.5. Moreover, mice in Sevo and Sevo+Dex were exposed to 2.5% sevoflurane and 97.5% air for 6 h in G15.5 while the mice in Con and Con+Dex were treated with 100% air for 6 h.

Preparation of Fetal Brain Slice and Immunofluorescence

A cesarean section was performed after the sevoflurane or air exposure and embryos were extracted. The fetal brains were then fixed in 4% paraformaldehyde for 12 h. After cryoprotection in 30% sucrose (wt/vol) for 12 h, embryonic brains were embedded with Tissue-TEK (O.C.T., Sakura Finetek, Tokyo, Japan) and was cryosectioned at 12 μm. For immunofluorescence, these sections were incubated in 10% goat serum in PBS for 2 h and permeabilized with 0.03% TritonX-100 in PBS. The following primary antibodies were used: PCNA (Abcam, ab5790, 1:400, Cambridge, MA, USA), Pax6 (Abcam, ab195045, 1:200, Cambridge, MA, USA), NeuN (Abcam, ab177487, 1:400, Cambridge, MA, USA), GFAP (Abcam, ab7260, 1:400, Cambridge, MA, USA), PH3 (Abcam, ab180577, 1:400, Cambridge, MA, USA), Ngn2 (Abcam, ab109236, 1:200, Cambridge, MA, USA), Tbr1 (Abcam, ab183032, 1:400, Cambridge, MA, USA). The following fluorescently conjugated secondary antibodies were used: goat anti-rat Alexa Fluor 594 (Abcam, ab150160, 1:500, Cambridge, MA, USA), and goat anti-rabbit Alexa Fluor 488 (Abcam, ab150077, 1:400, Cambridge, MA, USA). Cell nucleus was counterstained with DAPI (Abcam, ab285390, 1:400, Cambridge, MA, USA). Fluorescence images were acquired using a Leica TCS SP2 confocal microscope (Wetzlar, Germany).

Western Blots Analysis

The fetal brains were obtained by cesarean section immediately after treatment and were taken to protein analysis by western blot. The fetal brains were homogenated on ice in RIPA buffer (Cell Signaling, Boston, USA) plus protease inhibitor cocktail for 30 min. After centrifugation at 12,000 r.p.m. for 10 min at 4°C, the supernatant was removed and the protein concentration was measured by the BCA assay kit (Pierce technology Co., Iselin, NJ, USA).

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), the fetal brains protein homogenates were mixed with lane marker sample, separated by SDS-PAGE (12% gels), and then transferred onto a nitrocellulose membrane. After blocked in 5% skimmed milk for one hour, the membrane was then incubated with primary antibodies in Tris buffered saline for 12 hours at 4°C. The membrane was then washed by TBST and incubated with secondary antibodies for 1 hour at room temperature. Mouse or rabbit β-actin antibody (Cell signaling technology; 1:500, Boston, MA, USA) was used as the loading control. The protein bands were visualized with the Odyssey Imaging System (ODYSSEY CLx, LI-COR Biosciences, Lincoln, NE, USA). The primary antibodies were used as follows: anti-β-actin antibody (Cell Signaling Technology, 1:500, Boston, USA), anti-PCNA antibody (Abcam, ab29, 1:400, Cambridge, MA, USA), anti-anti-GSK-3β antibody (Abcam, ab32391, 1:400, Cambridge, MA, USA), anti-β-Catenin antibody (Abcam, ab8226, 1:400, Cambridge, MA, USA) and anti-CyclinD1 antibody (Abcam, ab16663, 1:400, Cambridge, MA, USA)

Analysis of Proportions of Cells Exiting the Cell Cycle with Cumulative BrdU Labeling

BrdU (Sigma, B5002, Merk KGaA, 70 g/Kg) was given intraperitoneally to G15.5 pregnant mice every 2 h over an 18-h period. The fetal brains were fixed in 4% paraformaldehyde and equilibrated in 30% sucrose (wt/vol) for 12 h at 4°C. The brains were then sectioned and processed to reveal BrdU as described in a previous study[6]. It has reported[6] that a pulse of BrdU injection results in different relative levels of BrdU labeling. Given that BrdU is the stuff of chromosomes and was diluted as cell mitosis, so the cells that undergo divisions presented relatively lighter BrdU labeling[6].

Statistical Analysis

The results were presented as means ± SEM. The student's *t*-test was performed for two-group comparisons and one-way ANOVA was used for multiple comparisons. *p*-values less than 0.05(*), 0.01(**) and 0.001(**) were considered statistically significant. Statistical analysis was performed using SPSS 22.0 software (IBM Corp., Armonk, NY, USA).

Results

Sevoflurane Inhibited the Self-Renewal of Neural Stem Cells in Fetal Brain

The proliferation of fetal brain was assessed immediately after the sevoflurane or air treatment. With the assessment of western blot, we found the expression of PCNA in fetal brain was significantly inhibited in the sevoflurane group [(Figure 1A-B), p=0.0351]. The result of the immunofluorescence of PCNA is consistent with western blot which showed a decrease of PCNA expression [(Figure 1C-D), p=0.0184]. As PCNA is a recognized marker for proliferation, these results indicated that maternal sevoflurane exposure during mid-gestation could inhibit the proliferation of fetal brain. We next studied the change in the number of Pax6$^+$ neural progenitor cells and found that the sevoflurane group exhibited a significant decrease in the size of neural progenitor pool [(Figure 1E-F), p=0.0406].

Sevoflurane Increased the Cell Cycle Exit of the Neural Stem Cells in Fetal Brain

The proliferation of neural progenitor cells is closely linked to cell cycle progression, so we next studied the change in the cell cycle phase. PH3 is an indicator of M-phases and we found a significant decrease in the expression of PH3 in

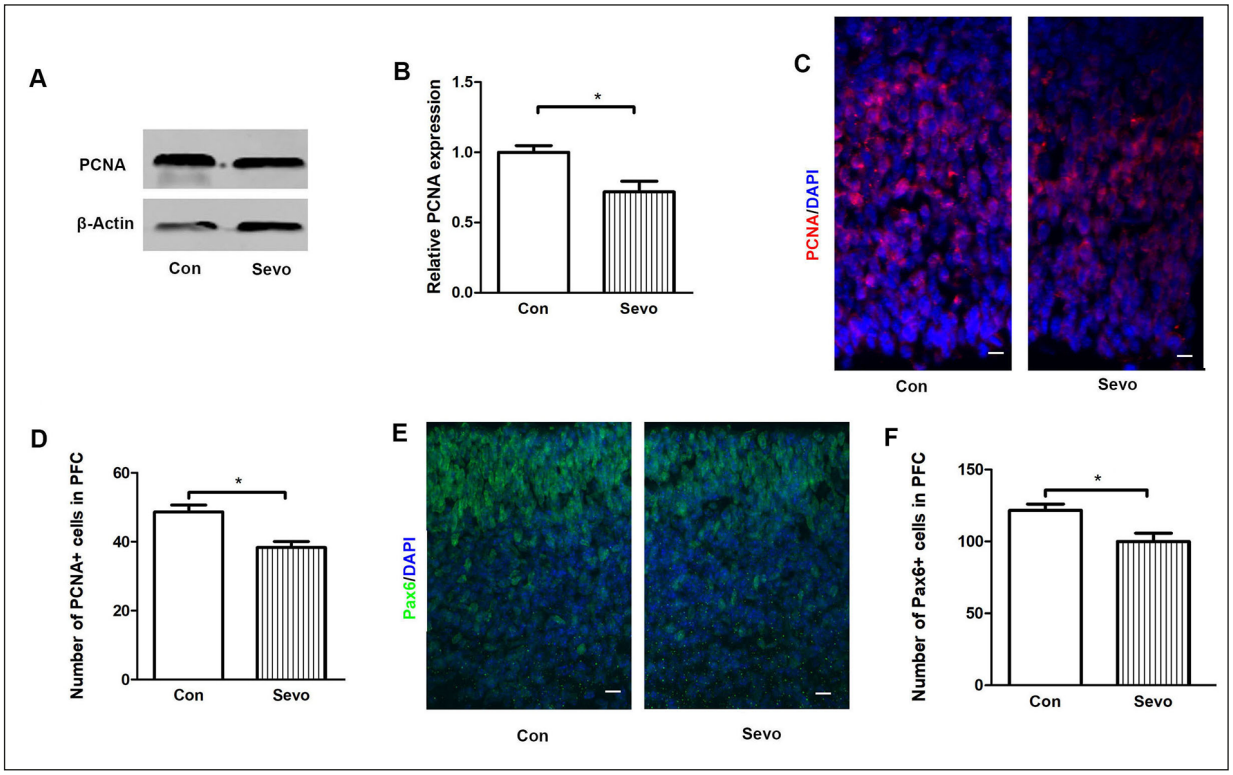

Figure 1. Prenatal sevoflurane exposure in third-trimester lead to abnormality in learning and memory ability. **A**, Western blot showed that maternal sevoflurane exposure inhibit ionthe expression of PCNA. **B**, Western blot quantification of PCNA (p=0.0351). **C**, PCNA (red) and DAPI (blue) in the fetal brain. Scale bars, 20 μm. **D**, Quantification of the PCNA$^+$ cells (p=0.0184). Data are expressed as the mean±SEM. *p<0.05 and **p<0.01. **E**, Pax6 (green) and DAPI (blue) in the fetal brain. Scale bars, 20 μm. **F**, Quantification of the Pax6$^+$ cells (p=0.0406)..

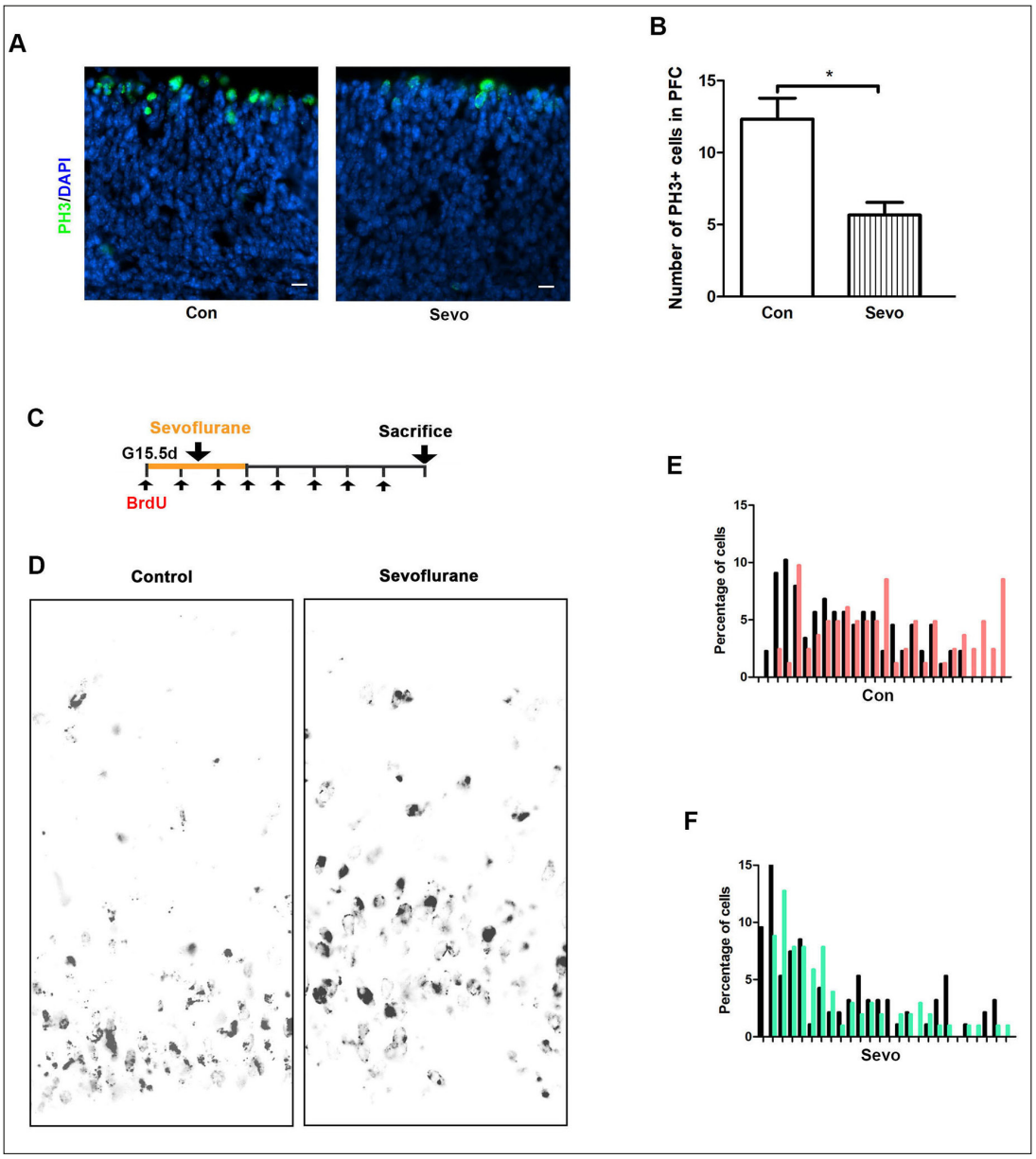

Figure 2. Sevoflurane increased the cell cycle exit. **A**, PH3 (green) and DAPI (blue) in the fetal brain. Scale bars, 20 μm. **B**, Quantification of the $PH3^+$ cells (p=0.0172). **C**, Schematic diagram of the timing of sevoflurane exposure, BrdU injection and cell cycle exit analysis. **D**, After an 18-h pulse with BrdU, heavily and lightly BrdU-labeled cell populations can be identified, and more lightly labeled cells are present in the Sevo group than in Con group (p=0.023). **E**, The percentage of the proliferating cells re-entering S-phase in the con group. **F**, The percentage of the proliferating cells re-entering S-phase in the Sevo group. $^*p<0.05$.

fetal brain after maternal sevoflurane exposure [(Figure 2A-B), p=0.0172]. We next studied the change of S-phase exit after maternal sevoflurane exposure. After 12.5 h pulses, the fetal brain in both Con and Sevo group showed bimodal distributions, but the proportions of lightly labeled cells were higher in the Sevo group than Con group, indicating there are less proliferating cells re-entering S-phase after sevoflurane exposure (Figure 2C-F).

Maternal Sevoflurane Exposure Inhibited Neurogenesis and Lead to Precocious Generation of Astrocytes in Fetal Brain

Given that neural stem cells are sequentially specified into neurons and gliocytes during the brain development, we next assess the change in differentiation after the treatment. The gliogenesis and neurogenesis were assessed 18 h after air or sevoflurane treatment (Figure 3A). We found a significant decrease in the number of $NeuN^+$ neu-

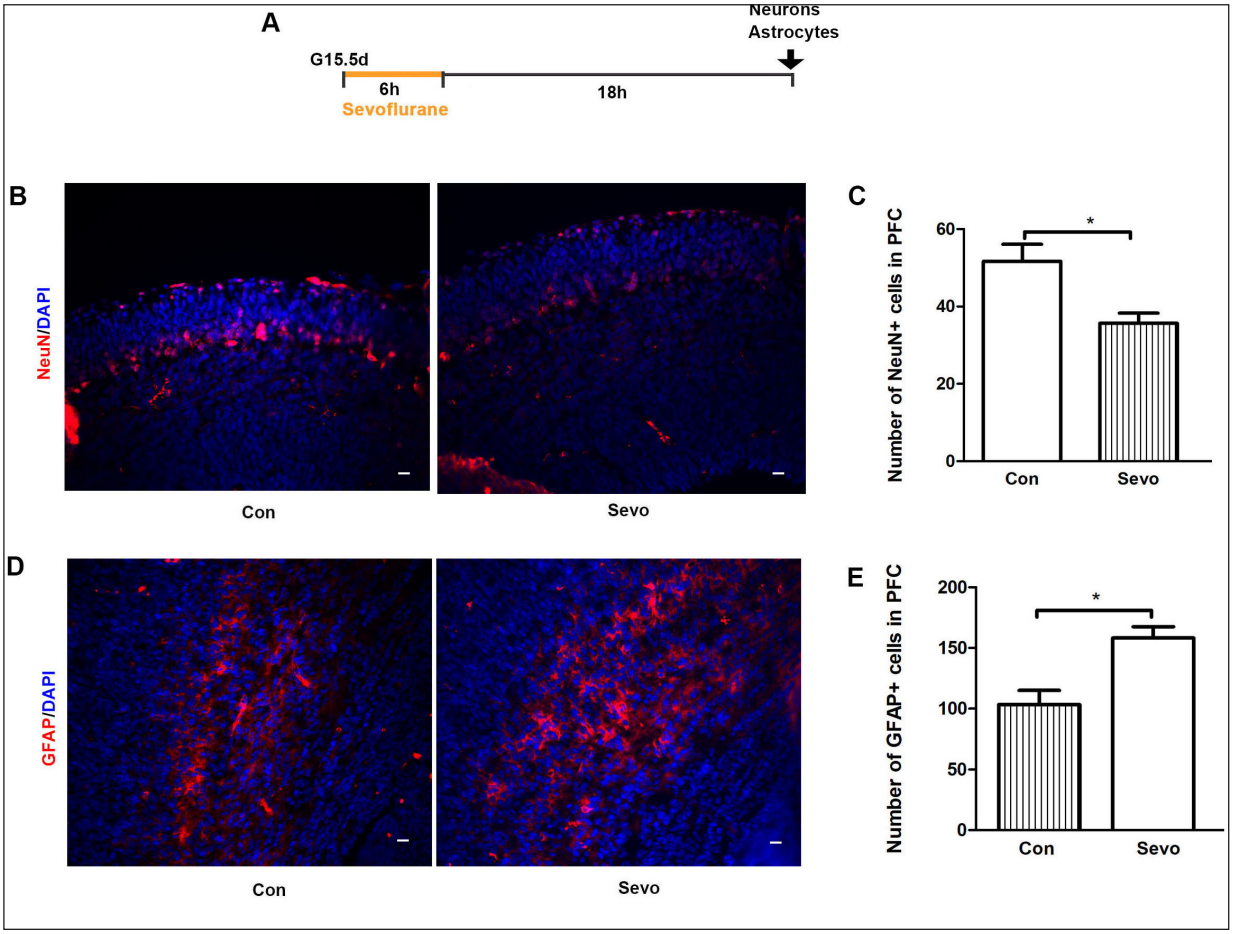

Figure 3. Maternal sevoflurane exposure inhibited neurogenesis and lead to precocious generation of astrocytes in fetal BRAIN. **A**, Schematic diagram of sevoflurane exposure and sacrifice to assess the differentiation of the fetal brain. **B**, NeuN (red) and DAPI (blue) in the fetal brain. Scale bars, 20 μm. **C**, Quantification of the NeuN$^+$ cells (p=0.0354). **D**, GFAP (red) and DAPI (blue) in the fetal brain. Scale bars, 20 μm. **E**, Quantification of the GFAP$^+$ cells. (p=0.0210). Data are expressed as the mean±SEM. $^*p<0.05$.

rons after sevoflurane exposure [(Figures 3B-C), p=0.0354]. However, the number of GFAP$^+$ astrocytes was increased in the sevoflurane group [(Figures 3D-E), p=0.0210]. Together, these results indicated that the neurogenesis was largely inhibited while the astrogliogenesis was more active in the fetal brains after maternal sevoflurane exposure.

Dexmedetomidine Blocked Sevoflurane-Induced Change in Proliferation and Differentiation in Fetal Brain

We next investigated whether dexmedetomidine has the neuroprotective effect on the sevoflurane-induced neurotoxicity. The mice were treated with physiological saline or dexmedetomidine from G12.5-G15.5. The change in proliferation were analyzed immediately after the sevoflurane or air treatment while the differentiation was analyzed 18 h later (Figure 4A). Results revealed an obviously decrease in the number of Pax6$^+$ neural progenitor cells, Tbr1$^+$ newborn neurons and GFAP$^+$ astrocytes after maternal sevoflurane exposure [(Figures 4B-G), p=0.0022, p=0.0034 and p=0.0134, respectively]. The decrease of neural stem cells, newborn neurons and astrocytes were ameliorated in the Dex group [(Figures 4B-G, p=0.0149, p=0.059 and p=0.0147, respectively].

Dexmedetomidine Could Mitigate Sevoflurane-Induced Inhibition in Wnt Signaling Pathway and the Decrease in the Expression of Cycind1 and Ngn2

The Wnt signaling plays a vital role in cell cycle regulation and is important in regulating the balance between the proliferation and differentiation in fetal brain. The expression of CyclinD1 could drive cell cycle from G0 to G1 phase and promote

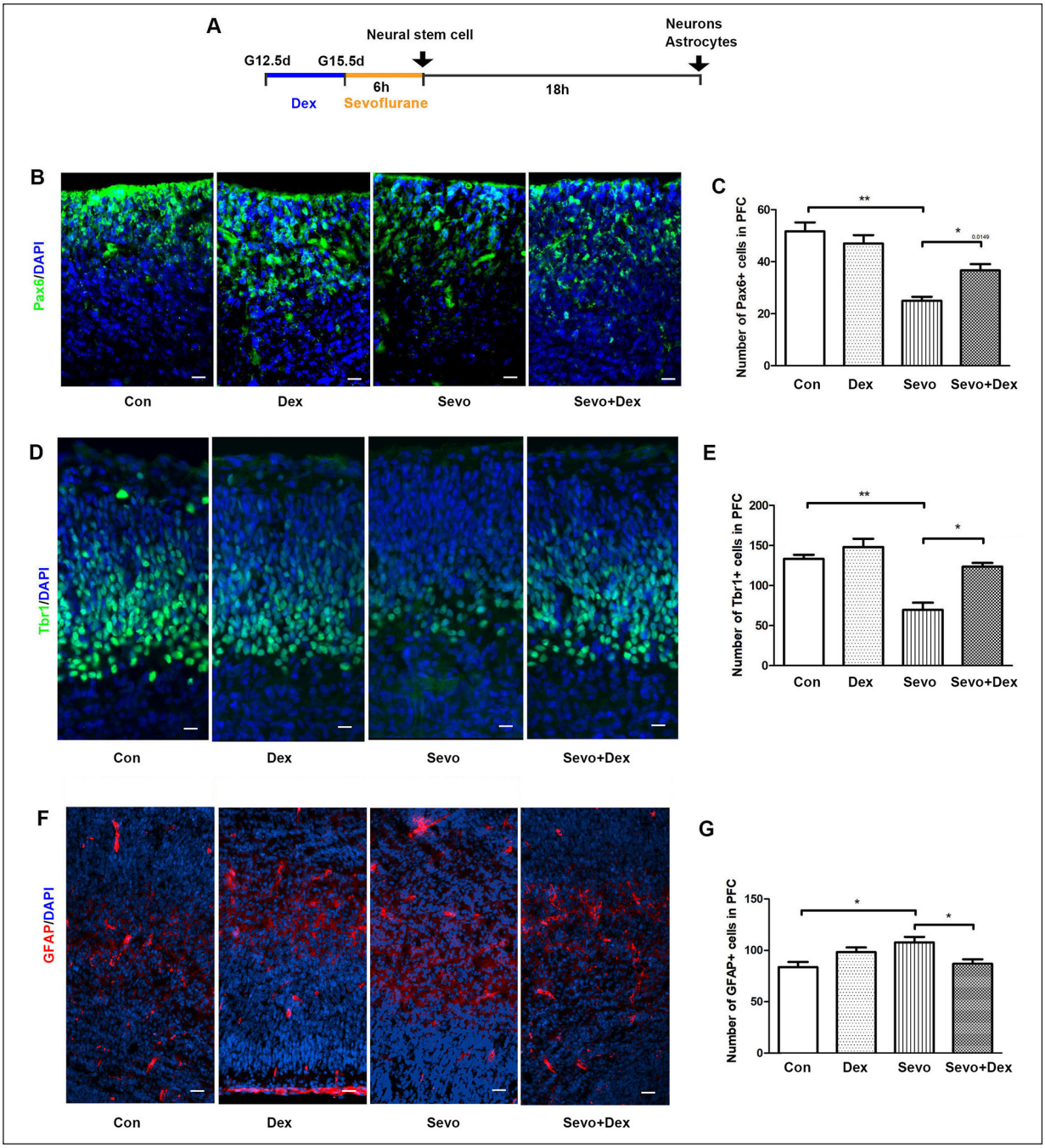

Figure 4. Dexmedetomidine blocked sevoflurane-induced change in proliferation and differentiation in fetal brain. **A**, Schematic diagram of dexmedetomidine administration, sevoflurane exposure and sacrifice to assess the proliferation and differentiation in the fetal brain. **B**, Pax6 (green) and DAPI (blue) in the fetal brain. Scale bars, 20 μm. **C**, Quantification of the Pax6$^+$ cells (Con *vs.* Sevo, p=0.0022 and Sevo *vs.* Dex p= 0.0149, respectively). **D**, Tbr1 (green) and DAPI (blue) in the fetal brain. Scale bars, 20 μm. **E**, Quantification of the Tbr1$^+$ cells (Con *vs.* Sevo, p=0.0034 and Sevo *vs.* Dex p=0.0059, respectively). **F**, GFAP (red) and DAPI (blue) in the fetal brain. Scale bars, 20 μm. **G**, Quantification of the GFAP$^+$ cells (Con *vs.* Sevo, p=0.0134 and Sevo *vs.* Dex p=0.0147, respectively). Data are expressed as the mean±SEM. $^*p$<0.05 and $^{**}p$<0.01.

proliferation. Ngn2 is important in the regulation of neurogenesis and astrogliogenesis in the fetal brain and has been reported to be suppressed directly by Wnt signaling pathway. To elucidate how dexmedetomidine mitigated the neurotoxicity of sevoflurane on fetal brain development, we next analyzed the activity of Wnt signaling pathway and the expression of CyclinD1 and Ngn2.

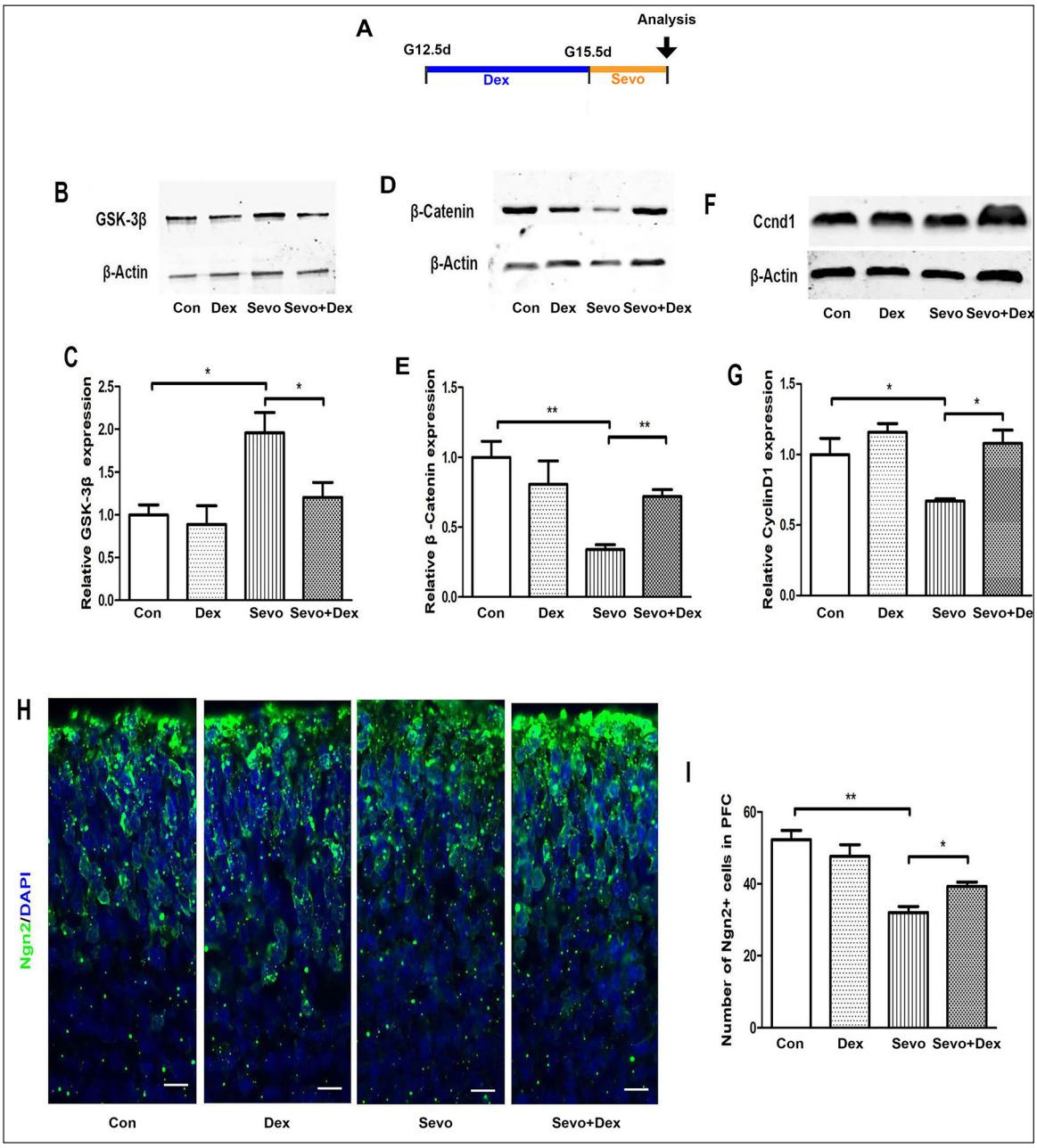

Figure 5. Dexmedetomidine could mitigate sevoflurane-induced change in Wnt-Ngn2 signaling pathway. **A**, Schematic diagram of dexmedetomidine administration, sevoflurane exposure and analysis. **B**, Western blot showed that the expression of GSK-3β is decreased after sevoflurane exposure which could be mitigated by dexmedetomidine. **C**, Western blot quantification of GSK-3β. (Con *vs.* Sevo, p=0.0112 and Sevo *vs.* Dex, p=0.0434). **D**, Western blot showed that the expression of β-Catenin is increased after sevoflurane exposure which could be mitigated by dexmedetomidine. **E**, Western blot quantification of β-Catenin. (Con *vs.* Sevo, p=0.0054 and Sevo *vs.* Dex, p=0.0033). **F**, Western blot showed that the expression of CyclinD1 is increased after sevoflurane exposure which could be mitigated by dexmedetomidine. **G**, Western blot quantification of CyclinD1. (p=0.0475). **H**, Ngn2 (green) and DAPI (blue) in the fetal brain. Scale bars, 20 μm. **I**, Quantification of the $Ngn2^+$ cells (p=0.0029). Data are expressed as the mean±SEM. $^*p<0.05$ and $^{**}p<0.01$.

The mice were treated with physiological saline or dexmedetomidine from G12.5-G15.5 and the change in the expression of the above proteins were analyzed immediately after the sevoflurane or air treatment (Figure 5A). Compared to the Con group, the expression of GSK-3β was significantly increased [(Figures 5B-C), p=0.0112] and the expression of β-Catenin protein was significant-

ly decreased in the Sevo group [(Figures 5D-E), p=0.0054]. Dexmedetomidine pre-administration could decrease the expression of GSK-3β and increase the expression of β-Catenin [(Figures 5B-E), p=0.0434 and p=0.0033, respectively], indicated that dexmedetomidine could activate the Wnt signaling pathway. We next assessed the change of Cyclin D1 and Ngn2 and found a significant decrease both in the expression of Cyclin D1 [(Figures 5F-G), p=0.0475] and Ngn2 [(Figures 5H-I), p=0.0029] after sevoflurane exposure. These changes were attenuated by maternal pre-administration of dexmedetomidine [(Figures 5B-I), p=0.0117 and p=0.0254 for CyclinD1 and Ngn2, respectively].

Discussion

With the rapid development of medical techniques nowadays, an increasing number of pregnant women are requiring surgery during pregnancy[3]. Maternal anesthesia exposure may lead to cognitive impairment in offspring and the mechanism is very complex, including neurogenesis, neuroinflammation, neuro-apoptosis and so on[7]. Normal human brain development begins in the early weeks of gestation and depends on well-organized proliferation and differentiation of neural stem cells[8]. Any adverse intervention factors such as anesthetics exposure could affect the development of the fetal brain. In our earlier studies[5], we have demonstrated that the offspring of pregnant mice exposed to sevoflurane developed cognitive dysfunction, but the underlying molecular mechanisms remains elusive. In the present study, we used a mice model to mimic maternal sevoflurane exposure and to investigate that the sevoflurane disturbs the balance of proliferation and differentiation and the neuroprotective effect of dexmedetomidine. Our data indicated that exposure of sevoflurane during the mid-gestation could inhibit neurogenesis and lead to premature generation of astrocytes in fetal brain. Furthermore, dexmedetomidine pre-administration could attenuate such change through activating the Wnt-Ngn2 signaling pathway.

In our study, pregnant mice were exposed to sevoflurane at G15.5, which is the critical period for neuronal proliferation and differentiation in rats[9]. Neural stem cell in the ventricular zone of prefrontal cortex proliferate to enlarge the neural stem cell pool and proceed the asymmetric division into immature neuronal cells after migrating to the subventricular zone[10]. Our results indicated that sevoflurane exposure at G15.5 could lead to a dramatic inhibition in the self-renewal of neural stem cells, which then lead to a significant decrease in the number of neural stem cell. Moreover, the number of the newborn neurons was decreased while the gliogenesis was more active in the sevoflurane group. The development of fetal brain is a continuous process and the abnormality caused by maternal sevoflurane exposure may bring huge impact to the structure and function. The prefrontal cortex has been reported to encode and regulate the highest cognitive functions in human brain[11], so the abnormality in the proliferation and differentiation of the brain may lead to cognitive dysfunction in offspring.

Cell cycle is a dynamic process which corresponds to a sequence of events including G1, S, G2 and M phase[8]. Some neural stem cells enter the cell cycle and proliferate to produce more neural stem cells while others exit the cell cycle and differentiate to produce neurons or astrocytes[12]. In this study, we found more neural stem cells withdrawing from the cell cycle to differentiate in the sevoflurane group. Given that the cell cycle progression is the key regulator of proliferation and differentiation in the neural stem cells during embryonic brain development[8], the increase in cell cycle withdrawal may lead to unbalance of fetal brain development.

Wnt signaling is a key regulator not only in neurogenesis but also in astrogliogenesis during brain development[8,13]. The Wnt signaling has been reported to be involved in multiple stages of fetal brain[13]. Cyclin D1 is one of the important cyclins that could regulate cell cycle transition from G1 to S phase[14]. Cyclin D1 is involved in the Wnt-mediated regulation of neurogenesis for that β-catenin could bound to the Cyclin D1 promoter and induced proliferation of neural stem cells[15]. Upon neurogenesis β-catenin could bind to Ngn2 gene promoter and promoted the production of neurons[15]. On the contrary, the Wnt signaling could inhibit astrogliogenesis by directly inhibition of Ngn2 and loss of β-catenin caused precocious production of $GFAP^+$ astrocytes[12]. In the current study, we found that the expression of GSK-3β was increased, and the amount of β-Catenin was inhibited, indicating that the activity of Wnt signaling was decreased after maternal sevoflurane exposure. The inhibition of Wnt signaling led to a suppression in the expression of Cyclin D1 and cell cycle withdrawal of neural stem cells. Moreover, the reduction of β-Catenin led to an increase of Ngn2 expression which, then, guided to

neurogenesis inhibition and the astrogliogenesis activation in the fetal brain.

Dexmedetomidine, a selective α2-adrenoceptor agonist, is now widely used in obstetrics anesthesia[16]. Dexmedetomidine has been reported to have protective and therapeutic properties with neurodegenerative disease such as Alzheimer's disease. Chronic but not acute dexmedetomidine treatments improved the cognitive functions in a dose-dependent manner. We treated the pregnant mice with dexmedetomidine with the dose of 50 μg/Kg. In the current study, we found that the maternal dexmedetomidine pre-administration could reduce the abnormalities of proliferation and differentiation in fetal prefrontal cortex caused by sevoflurane. After a chronic administration of dexmedetomidine, there was an obvious increase in the number of neural progenitors, newborn neurons as well as a significant decrease in the number of astrocytes. The beneficial effect of dexmedetomidine is accompanied by the activation of Wnt signaling, elevated levels of CyclinD1 and Ngn2. The present findings suggest that chronic dexmedetomidine ameliorates sevoflurane-related neurogenesis through inhibiting the Wnt signaling pathway in the fetal brains.

Limitations

However, it should be noted that our research has some limitations. We selected G12.5-G15.5 mice instead of an exact gestation age. Considering that the fetal mice brain develops rapidly during pregnancy, so the numbers and types of the neuronal cells may be different between the fetal mice brain of different gestational mice.

Conclusions

Our research indicated that maternal sevoflurane exposure could activate the Wnt signaling pathway and lead to the abnormal development in fetal mice brain. Furthermore, the chronic administration of dexmedetomidine could inhibit Wnt signaling and rescue the neurodevelopmental deficits in fetal brain. Together, these observations further verify the neurotoxicity of sevoflurane to fetal mice brain development and provide new insights the neuroprotective role of dexmedetomidine. We suggest doctors to consider the neurotoxicity of anesthesia to fetal brain development during gestation. Moreover, maternal dexmedetomidine administration may be helpful to the fetus if the anesthesia exposure is necessary.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgments

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Ethics Approval

All procedures were approved by the Animal Care and Use Committee of Qingdao University.

Authors' Contribution

Ruixue Song designed the study and performed the experiment; Rong Wang contributed significantly to data acquisition and statistical analysis; Guishen Miao researched literatures and wrote the manuscript; He Dong is the guarantor of integrity of entire study and is responsible for the manuscript review.

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