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Original article

Isoflurane inhibits embryonic stem cell self-renewal through retinoic acid receptor

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ABSTRACT

The commonly used inhalation anesthetic isoflurane could permeate rapidly through the placental barrier and induce toxicity to the central nervous system of the developing fetus. However, the effects of isoflurane ***in utero*** during early gestation are unknown. We therefore treated pregnant mice with 1.4% isoflurane for 2 h per day for three days at day3.5 (E3.5) to day6.5 (E6.5) to investigated the toxicity of isoflurane. Pregnant mice were executed and the fetal mice were weighed and observed. Mouse ESCs (E14) was exposed to 2% isoflurane for 6 h. Twenty-four hours later, self-renewal was examined with AP staining. Effects of isoflurane on the expression of RAR-γ were examined using Western blot. As a result, anesthesia with 1.4% isoflurane for 2 hour per day for 3 days reduced fetal growth and development. Isoflurane decreased self-renewal and the expression stemness genes (Nanog, Oct4, Sox2, and Lin28) in mESCs. Vitamin A attenuated the effects of isoflurane inducing self-renewal inhibition. In summary, Anesthesia with 1.4% isoflurane for 2 h per day for 3 days reduced fetal growth and development. Moreover, isoflurane inhibits mESCs self-renewal through retinoic acid receptor.

1. Introduction

More and more pregnant women nowadays require non-obstetric surgery [1]. In USA, about 75,000 pregnant women undergo non-obstetric surgery each year [2]. A clinical research found that anesthesia could be an increased risk for the development of brain [3]. In animal studies, the commonly used inhalation anesthetic isoflurane induced neuroinflammation and inhibited neurogenesis in the brain tissues of fetal mice and caused subsequent learning and memory impairment after multiple exposures [4]. Recent study found that long term isoflurane treatment inhibited proliferation, differentiation, and survival in human neuroprogenitor cells [5,6]. Because isoflurane could permeate rapidly through the placental barrier and induce toxicity to the central nervous system of the developing fetus, the effect of inhalation anesthetic on the development of fetal mice need to be determined. Therefore, in this study, we used mouse embryonic stem (mES) cells as a model to exam the effect of inhalation anesthetic on the development of fetal mice partly.

Stem cells are undifferentiated biological cells that can differentiate into specialized cells and can divide to produce more stem cells [7]. In a developing embryo, stem cells can differentiate into all the specialized cells—ectoderm, endoderm and mesoderm [8]. Self-renewal exists to ensure that a stem cell population is maintained [9]. A stem cell divides into one mother cell that is identical to the original stem cells, and another daughter cell that is differentiated. Or one stem cell develops into two differentiated daughter cell, another stem cell undergoes mitosis and produces two stem cells identical to the original [10]. This suggests that we can use embryonic stem cells as a model to investigate the effect of inhalation anesthetic on the development of embryo partly.

The retinoic acid receptors (RAR α,β,γ) and the retinoid X receptors (RXR α,β,γ) areligand-activated transcription factors. RAR heterodimerizes with RXR andbinds to specic DNA sites, while RXR also can bindDNA as a homodimer [11–13]. Vitamin A was discovered almost 100 years ago. Retinol is the alcohol form of vitamin A. Vitamin A/retinol come inside the cytoplasm and convert into retinoic acid by two sequential oxidation steps. Retinoic acid is then transported to the nucleus where it binds to retinoic acid receptor (RAR) and retinoid X receptor (RXR). Vitamin A/retinol supports the self-renewal of stem cells. Recent study

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revealed that mGSCs proliferated faster in retinol medium and exhibited strong proliferation and colonization. Treatment of human ESCs with vitamin A/retinol resulted in excellent morphology of the undifferentiated colonies. These studies suggested that RAR/ RXR may regulated the the self-renewal of stem cells.

In this study, we treated mES cells with isoflurane to examine the potential repression of isoflurane on the self-renewal and also assessed the effects of isoflurane on the activity of RAR/RXR in mouse ES cells.

2. Materials and methods

2.1. Animals

All animal experiments were approved by the Animal Care Committee of Shanghai East Hospital, which is affiliated with Tongji University. Two months aged C57BL6/J mice (SLAC laboratory animal, Shanghai) with one quarter males and three quarters females were housed in clean cages. The male and female mice were mixed at the ratio of 1–3 to mate overnight and mating was confirmed by the presence of a vaginal plug in the following morning. Date of plug positivity was designated as day 0.5 (E0.5) and pregnant mice were treated with 1.4% isoflurane for 2 h per day for three days, from day3.5(E3.5) to day6.5 (E6.5). At day18 (E18), pregnant mice were executed by cervical vertebra luxation and the fetal mice were harvested by cesarean section. The fetuses were then weighed and observed.

2.2. Cell culture

E14 mES cells, purchased from Institute of Biochemistry and Cell Biology in Shanghai of China (SCSP-204), were seeded in plate pre-coated by 0.1% gelatin in DMEM (Gibco) containing 15% ES-FBS (Gibco), glutamine (1:100; Invitrogen), NEAA (1:100;Invitrogen), B-ME (1:550;Invitrogen), and Lif (1:10000; Invitrogen) at a density of 7×10^4 cells per well in six well plates, and cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. Cell treatment

Isoflurane group were exposed to 2% isoflurane, 5% CO_2 plus 21% O_2 and control group conditions included 5% CO_2 plus 21% O_2 for 4,6 h, as previously described. A DragerVamos gas analyzer (Drager, Germany) was used to monitor the concentration of CO_2, O_2, and isoflurane [5]. In some experiments, cells were treated with 1 μmol/L Vitamin A at 6 h prior to isoflurane exposure.

2.4. Gene set enrichment analysis (GSEA)

Gene set enrichment analysis (GSEA) was used to determine whether genes of RAR signaling showed statistically significant, concordant correlation with either of the two groups. The RAR signaling signature, obtained from the MSigDB database, was used to characterize RAR characteristic. Default parameters were used. The phenotype was permutated 1000 times. A heat map was generated by color-coding gene expression intensities. The color code showed higher (red) or lower (blue) expression relative to the mean expression of that gene in all samples.

2.5. RT-PCR and Real-Time PCR

Total RNA was extracted using Trizol reagent (Sigma), and reverse-transcribed using M-MLV Reverse Transcriptase (Promega) using primers published in a previous study[5]. The real-time quantitative PCR included 40-cycles of amplification. Expression of target genes ($2^{\Delta DCt}$) was normalized against endogenous GAPDH. Quantitative RT-PCR was carried using an Mx3000P system (Stratagene).

2.6. Western blotting analysis

Cell lysate was re-suspended using 5 × loading lysis buffer (250 mM Tris–HCl (pH6.8) 5% DTT, 10% SDS, 0.5% bromophenol blue 0.025 g, 50% glycerine The membrane was then incubated with a primary antibody against Lin28 (Abcam) or GAPDH (Cell Signaling Technology). Signals were visualized by enhanced chemiluminescence (ECL, Thermo).

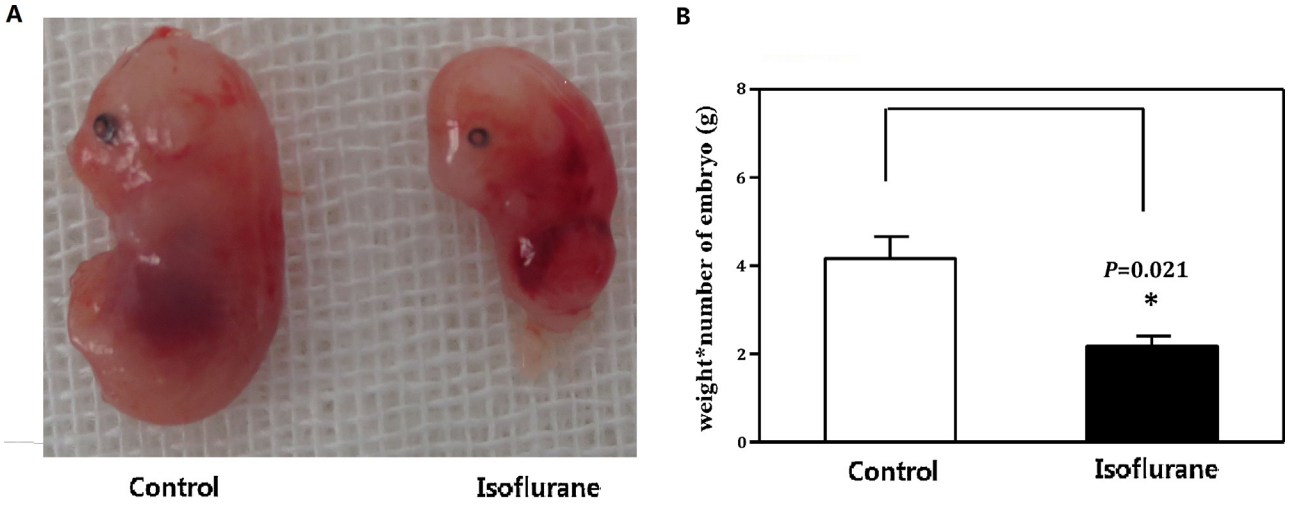

Fig. 1. Anesthesia with 1.4% isoflurane for 2 h daily for 3 days reduced fetal growth and development, especially the brain development.
(A) Anesthesia with 1.4% isoflurane for 2 h daily for 3 days reduced fetal growth and development. Ctrl means the fetal mouse from pregnant mouse without treatment. isoflurane means fetal mouse from pregnant mouse treated with 1.4% isoflurane.
(B) Isoflurane reduced fetal growth as compared with fetal weights* number of fetus between control group and isoflurane group. Data shown are means ± SD ($n = 20$). *$p < 0.05$.