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Research Article

Isoflurane Inhibit the Self-Renewal Capacity of Human Embryonic Stem Cells and Urge them to Differentiate

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Abstract

Background: The inhalation anesthetics, owning the character of lipophilic, could permeate through the placental barrier and the blood-brain barrier rapidly, which draws increasing public attention on the embryotoxicity and neurotoxicity of inhalation anesthetic in the developing fetus. The potential toxicity of commonly used inhalation anesthetic isoflurane on the human embryonic stem cells (ESCs) is becoming a significant health issue.

Methods: Human ESCs which is derived from the inner cell mass (ICM) were treated with 2% isoflurane for 6h. At the same time, the control group was untreated. We observe the growth and detect the mRNA levels of stemness markers of ESCs in two groups.

Result: Compared with control group, the self-renewal capacity of human ESCs in isoflurane group was distinctly inhibited. Moreover, cells treated with isoflurane appeared differentiation prior to the control group.

Conclusion: The observed phenomena suggest that isoflurane inhibits the self-renewal capacity of human embryonic stem cells derived from ICM and makes them differentiation in advance.

Introduction

With the improvement in medical treatment and the advancement of anesthetic and surgical technique, the potential embryotoxicity and neurotoxicity of anesthesia drugs is drawing more and more attention of the healthcare and the public. In recent decades, there have been many reports about this issue. Related studies could not be carried out directly on human beings because of the involved ethics. What's more, many inevitable factors, such as the surgical operation, patients' pathophysiological condition, the interactions among anesthesia drugs and so on, made the findings of the clinical investigations lack of persuade. Therefore, we use human ESCs to do some basic researches about the potential embryotoxicity and neurotoxicity of anesthesia drugs. We hope our research could provide reasonable guid-

ance for drugs-use in clinic.

Statistics shows that between 0.75% and 2% of pregnant women require non-obstetric surgery [1]. However, about 75000 pregnant women have to undergo non-obstetric surgery every year in United States [2]. Owing to the physiological characteristics of pregnant women, the subject of the effects of anesthesia drugs upon pregnant women and their fetuses during the non-obstetric surgery is urgent to probe into. Given the limitation of clinical trials, we adopted human ESCs as the experimental model to explore the embryotoxicity of anesthesia drugs. The ESCs derived from inner cell mass of blastocysts are characterized by self-renewal and pluripotency [3]. For this research, we applied them to simulate the embryo and could be more intuitive to study the potential embryotoxicity of anesthesia drugs.

Isoflurane is one kind of commonly used inhalation anesthetics. It was widely used in clinic with rapid inducing and recovery effects, a certain degree of muscle relaxation function and less adverse effects and complications. Isoflurane could permeate through the placental barrier and the blood-brain barrier rapidly because of its lipophilic character. Previous study suggested that isoflurane could significantly inhibit fetal growth in pregnant mice [4]. Furthermore, rats exposed to isoflurane in utero during early gestation is behaviorally abnormal as adults [5]. Recent research shows that isoflurane could decrease the ability of self-renewal and neuronal differentiation of mouse embryonic stem cells [6]. In view of the above, we speculate that isoflurane have potential toxicity effects on embryonic development. Therefore, in this experiment we try to investigate the effects isoflurane exerting on human embryonic stem cells.

Materials and Methods

Cell culture

Human embryonic stem cells (H9 and H1 lines, passages 18–35), provided by the WiCell Institute, are cultured on irradiated mouse embryonic fibroblasts (MEFs) feeder layers. Usually, they are passaged every five or six days. Cultured medium is sterile hybrid with 392.5ml DMEF/F12 (Gibco), 100mL Knockout serum replacer (Gibco), 5ml MEM nonessential amino acids solution (Invitrogen), 2.5ml of 200mM L-glutamine solution (Invitrogen), and 3.5μl 14.3M β-mercaptoethanol (Invitrogen). Prepared medium is stored at 4°C no more than one week. Ten minutes before starting cell-passage, warm up DMEM/F12, dispase (Gibco) and the culture medium in a 37°C water bath. Collect the intact ESCs colonies for passage every time. ESCs are cultured as previously described [7,8]. The 6-well plates containing ESCs are placed in a humidified incubator with 5% CO_2 at 37°C. Cells in the isoflurane group were treated with 2% isoflurane for 6h as described [9,10]. Observe the growth of ESCs in two groups under inverted microscope and grab images every day.

RT-PCR and Real-Time PCR

Extracted total RNAs from cells using Trizol reagent (Sigma). Then, reverse-transcribe RNA using M-MLV Reverse Transcriptase (Promega). During the real-time PCR analysis, we applied an MX3000P Stratagene PCR machine to amplify for 40 cycles using 2×RT-qPCR Mix (BioRed). The amount of target gene expression (2ΔΔCt) was normalized via the endogenous GAPDH. Primer sequences for our study were referred to previous paper [11].

Results

Isoflurane inhibit the self-renewal capacity of human embryonic stem cells and urge them to differentiate

In order to study the influence of isoflurane on human embryonic stem cells, we implement anesthesia with 2% isoflurane for 6h on hES cells to mimic the growth in vivo. We treated the cells with 2% isoflurane for 6h because some previous studies have found that isoflurane induced elevation of cytosolic calcium levels, caspase-3 activation, Aβ accumulation, and mitochondrial dysfunction in the H4 cells and neurons with 2% isoflurane treated for 6h [12-15]. In our experiment, we made the human ESCs in the isoflurane group exposed in 2% isoflurane, 5% CO_2 plus 21% O_2 for 6h. At the same time, the control group was exposed to 5% CO_2 plus 21%O_2. Cultured cells for another 4 days in the same incubator (37°C), we compared the cell communities between control group and isoflurane group. Interestingly, we found the growth of ESCs in the isoflurane group was repressed and the cells appeared differentiation in advance (Fig 1A).

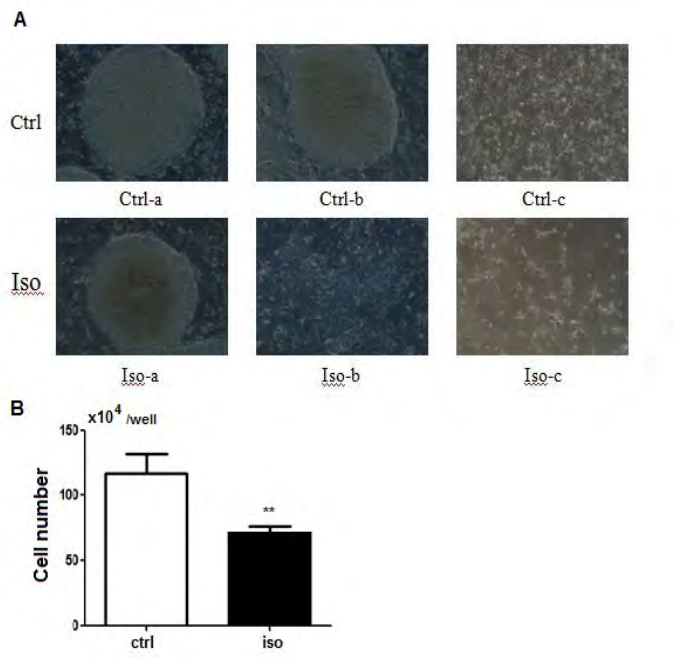

Figure 1. Isoflurane inhibits the self-renewal capacity of human ESCs and urges them to differentiate.

A. The morphology of hESCs treated by isoflurane and control group. Crtl means the ESCs without treatment. Iso means the ESCs treated with 2% isoflurane for 6h. The images ware observed at the first, the fifth and the seventh day after cells-passage, respectively.

B.The statistics of cell number of hESCS treated by isoflurane and control group on day 4. Ctrl means the ESCs without treatment. Iso

means the ESCs treated with 2% isoflurane for 6h. Data shown are means±SD (n=5), **P<0.01, two-tailed Student's t-test.

Crtl means the ESCs without treatment. Iso means the ESCs treated with 2% isoflurane for 6h. The images ware observed at the first, the fifth and the seventh day after cells-passage, respectively.

Additionally, we had found that the cell number in the isoflurane treating group was less than control group on day 4, which may showed the toxicity effect of isoflurane (Fig 1B). We further detected the mRNA levels of stemness markers (including Oct4, Sox2, Nanog, Lin28) and found the levels were down-regulated at various degree (Fig 2A). We also performed the differentiation and detected the expression of the markers of three germ layer. AFP is the marker of indicating endoderm. T is the marker of indicating mesoderm. Nestin is the marker of indicating ectoderm. We found that the isoflurane influenced the self-renewal, promoting the differentiation by detecting the upregulation of the expression of markers (Fig 2B).The results suggested that the pluripotency of the isoflurane-treated human ESCs was attenuated. Accordingly, we confirmed that isoflurane could inhibit the self-renewal capacity of human ESCs and urge the cells to differentiate.

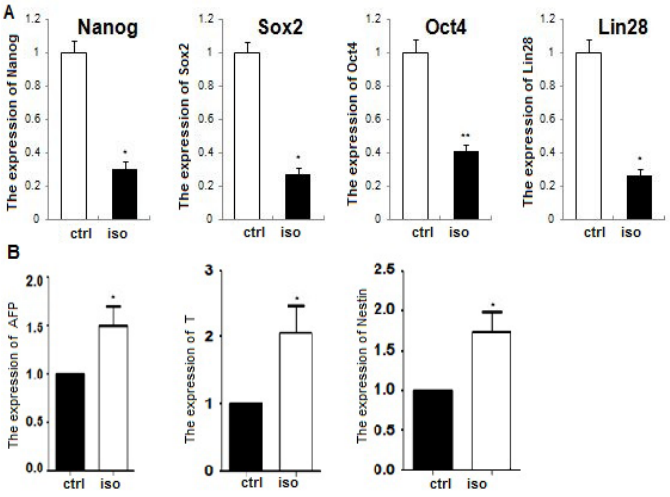

Figure 2. Isoflurane represses the self-renewal and promoted the differentiation of human ESCs .

A. Isoflurane downregulated the expression of stemness markers detected by qPCR. Ctrl means the ESCs without treatment. Iso means the ESCs treated with 2% isoflurane for 6h. Stemness markers of Nanog, Sox2, Oct4, Lin28 in ESCs is detected by RT-PCR. Data shown are means±SD (n=3), *P<0.05, **P<0.01, two-tailed Student's t-test.

B. Isoflurane upregualted the expression of three germ layer markers detected by qPCR. Ctrl means the ESCs without treatment. Iso means the ESCs treated with 2% isoflurane for differentiation. Data shown are means±SD (n=5), *P<0.05 two-tailed Student's t-test.

Ctrl means the ESCs without treatment. Iso means the ESCs treated with 2% isoflurane for 6h. Stemness markers of Nanog, Sox2, Oct4, Lin28 in ESCs is detected by RT-PCR. Data shown are means±SD (n=3), *P<0.05, **P<0.01, two-tailed Student's t-test.

Discussion

Every year, there are a large amount of pregnant women exposed to inhalation anethestics for non-obstetric surgery [1]. Moreover, the population presents a rising tendency for the increasing laparoscopic procedures and fetal surgery [16]. With the increasing of non-obstetric surgery among pregnant women, the embryotoxicity of inhalation anesthesia has drawn more and more attention. Many studies have reported that rats exposed to isoflurane in utero during early gestation would appear learning and memory impairment [17] and also behaviorally abnormal as adults [18,19]. And according to these researches, all of the phenomena related to the lipotropy of inhalation anesthetics. In a previous research [6], clinical dosage of isoflurane (2%) can repress the mouse ESCs self-renewal by down-regulating pluripotent genes (such as Sox2, Oct4, et al.). The finding is consistent with those of Culley who showed that isoflurane could decrease the self-renewal capacity of rat cultured neural stem cells [20]. All the above confirmed that isolfurane may have potential toxicity on embryonic development. However, nearly studies have demonstrated the effects of isoflurane imposing on human embryonic stem cells.

To further investigate whether isoflurane could exert the same effects on human ESCs, we carried out this research to observe them. In this study, human ESCs were exposed to isoflurane at a clinical relevant concentrate (2%) to simulate pregnant women undergoing long time non-obstetric. Human embryonic stem cells with two grant characters of self-renewal and differentiation come from ICM and play a significant role in embryonic development. We found ESCs in isoflurane-treated group self-renewal was repressed. Also, the mRNA levels of their stemness markers Oct4, Sox2, Nanog, Lin28 were down-regulated. At the same time, we observed that the isoflurane-treated group stepped into differentiation stage in advance of the control group.

Isoflurane is a clinically common used inhalation anesthestic and an important adjuvant agent in general anesthesia. Meanwhile, isoflurane with the character of lipotropy can permeate through the placental barrier and the blood-brain barrier rapidly. Therefore, the potential toxicity of isoflurane has inevitably become a focus of medical practitioner and the public. Just because of these reasons, our findings in this study showed very important clinical significance.

In summary, isoflurane repressed the self-renewal capacity of human embryonic stem cells, down-regulated related stemness genes, made them appear differentiation in advance. However, the potential mechanism of the effect of isoflurane on human ESCs is still largely unknown and needed to be further researched.

Acknowledgments

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Conflicts of Interest

No conflicting relationship exists for any author.

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