Sevoflurane inhibits Human umbilical vein endothelial cells migration by up-regulating VE-cadherin expression

Yuanqing Sun

the international peace maternity and child health hospital

Zifeng Xu (✉ xuzf@shsmu.edu.cn)

the international peace maternity and child health hospital, School of medicine, Shanghai Jiao Tong University

Tao Xu

the international peace maternity and child health hospital

Hui Xu

the Ninth People's Hospital

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Abstract

Background Sevoflurane is a commonly used inhalation anesthesia and is famous for rapid onset of action, less metabolism in vivo and fast recovery. The aim of this study is to elaborate whether sevoflurane inhibits Human umbilical vein endothelial cells (HUVECs) migration function.

Methods In vitro experiments, the HUVECs were divided into four groups randomly, and were exposed to 2% sevoflurane, refer to 1.6 minimal alveolar concentration (MAC), respectively for 0.5h, 1h, 2h and the first group was the control group which exposed to the same gas environment with other three groups but only without sevoflurane. After sevoflurane exposure, HUVECs were conducted the scratch assay.

Results The results suggested that the HUVECs exposed to 2% sevoflurane for 2 h were more obviously inhibited on the migration distance during 12 h after scratched than the control group. Quantitative PCR results suggested that the HUVECs exposed to 2% sevoflurane for 2 h expressed more vascular endothelial cadherin (VE-cadherin) than the control group, with statistic difference. However, other scratch assay and quantitative trials suggested that the HUVECs which were transfected with VE-cadherin siRNA and exposed to 2% sevoflurane for 2 h had no significant difference with the control group on the migration distance and the expression of VE-cadherin.

Conclusion These results suggested that sevoflurane inhibited the HUVECs migration function by up-regulating VE-cadherin expression, and may have an adverse effect on the normal functions of vascular endothelial cells.

Background

Sevoflurane is a widely used inhalation anesthetic in clinical, and it offers some advantages over other inhalation anesthetics, such as low solubility, fast induction and faster recovery with long procedures[1]. However, its exact pharmacological mechanism is still unclear. It is well known that normal vessel functions depend on normal vascular endothelial cells function and structure, and vessel functions involve in much physiological process and pathologic process. For instance, the incidence of non-obstetric surgery during pregnancy was 0.48%, mostly performed during the second trimester (44%) and under general anesthesia (81%)[2]. There are many studies that have reported the adverse impact of sevoflurane exposure during mid-pregnancy on the nervous system of offspring rats[3,4]. However, the effect of sevoflurane exposure during pregnancy on the blood vascular system in fetus, especially on the umbilical vein which provides enough oxygen and rich nutrients for fetus, has been rarely reported. Another example, the free skin flap transplantation is commonly used in the plastic and reconstructive surgeries, during which the transplanted blood vessels provide adequate blood supply to the free skin flap to guarantee its survival. There are many factors that impact the survival of the free skin flap, such as tobacco use, arteriovenous loop, surgical technique, donor/recipient sites, et al[5,6]. Smooth anastomosis of blood vessels to ensure the normal structure and function of vascular endothelial cells is the crucial step in these surgeries. In the procedure of plastic and reconstructive surgeries, patients need to receive

relatively long-time sevoflurane anesthesia. But the definite effects of sevoflurane on vascular endothelial cell function has been rarely reported so far and whether sevoflurane affects the prognosis of free skin flap transplantation by disturbing the transplanted vessels functions is unknown.

Vascular endothelial-cadherin (VE-cadherin) belongs to the cadherin family, and it is extensively considered to be specific for vascular endothelia in which it is either the sole or the predominant cadherin[7]. Some recent studies have indicated that immature endothelial cells show two different types of VE-cadherin distributions, they adjust HUVECs to two opposite states, and the coexistence of these two different distributions of VE-cadherin in endothelial cells is required for normal functions of vascular endothelial cells[8]. Junctions between endothelial cells play a pivotal role in the regulation of vascular endothelial functions, such as angiogenesis, proliferation, vascular endothelial permeability, and involve in some pathologic process, like atherosclerosis[9]. VE-cadherin plays an important role in angiogenesis, some drugs can inhibit tumour-induced angiogenesis by down-regulating VE-cadherin expression[10,11]. Vascular endothelial cells proliferation function is regulated by VE-cadherin when cell-to-cell contact, and the inhibition of cell growth is density dependent[12]. Andes virus downregulated VE-cadherin expression to disrupt endothelial cell barrier and increase vessel permeability[13]. VE-cadherin also involved in the mechanisms of atherosclerosis[14,15]. Because of the important role of VE-cadherin in the regulating of vascular endothelial cell functions, we considered seriously if VE-cadherin involves in the mechanisms of the inhibition migration of HUVECs by sevoflurane.

HUVECs are widely used in vitro model to study vascular endothelial cell functions because they come from human umbilical cord and can guarantee homology[16]. In our study, we used monolayer HUVECs to represent the umbilical vein in pregnant women who are under sevoflurane anesthesia or simulate transplanted blood vessels in the free skin flap transplantation surgeries, we selected HUVECs as a experiment subject and we tried to investigate whether sevoflurane could inhibit HUVECs migration by changing the expression of VE-cadherin and further to affect the normal functions of vascular endothelial cells.

Methods

Materials and Apparatus

HUVECs (GNHu39, purchased from cell bank of Chinese Academy of Sciences, China). Fetal bovine serum (FBS) (Gibco. USA), Dulbecco's modified eagle medium (DMEM) (Gibco. USA), Penicillin/Streptomycin (Gibco. USA), Pancreatin (Gibco. USA). Sevoflurane (Abbott Laboratories. USA). RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher.K1622. USA), Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher.K0221. USA), Trizol reagent (Invitrogen, Carlsbad, CA), RNeasy kit (Qiagen, Dusseldorf, Germany), Quantitative PCR instrument (Bio-RAD. USA). Flow cytometer (BD. USA). BCA Protein Assay Kit (pa115-01, TIANGEN. China), Wide range color pre-stained protein marker (MP206,

TIANGEN, China), HRP-DAB substrate reagent kit (PA109, TIANGEN, China), GAPDH antibody (GTX17307, TIANGEN, China). Lipofectamin RNAiMAX reagent (Invitrogen, USA)

HUVECs treatment

HUVECs treatment was performed as previously described[17]. Sterile technique was abided during all experiment operations. Vascular endothelial cells were cultured in DMEM which contains 1% penicillin/streptomycin, 5% FBS, and 1% endothelial cell growth supplement, and in a humidified atmosphere contains 5% CO₂ and 95% air at 37°C.

Sevoflurane exposure

Sevoflurane exposure was performed as previously described[18]. A sealed plastic box was prepared in advance, it has an air inlet and an outlet. A Datex infrared gas analyser (Puritan-Bennett, Tewksbury, MA, USA) was necessary to continuously monitor the delivered oxygen, CO₂ and sevoflurane concentrations. In the section of sevoflurane exposure, 21% oxygen and 5% CO₂, 2% sevoflurane or not, were delivered from an anesthesia machine to a sealed plastic box, and the sealed box was placed in an abacteria incubator at 37°C all the time. Cells were cultured in 6-well plates and when cells grow to 90%-95% confluence, the 6-well plates without lips were put into the sealed plastic box (the box was sterile also) to receive sevoflurane exposure. We treated the cells with 2% sevoflurane for 0.5 h, 1 h, 2 h respectively in serum free media. The first group was the control group which exposed to the same environment with other three groups but without sevoflurane.

siRNA transfection

HUVECs were planted into 6-well plates at a density of 2×10^5 cells/well and then cultured in a humidified atmosphere contains 5% CO₂ and 95% air at 37°C. Cell suspension was harvested in serum free media, and cell count was used to maintain the density of 8×10^4 /ml for transfection. Added 1.5 µl VE-cadherin siRNA, 500 µl Optimem and 5 µl RNAMAX in per plate and incubated in room temperature. Added 2.5 ml HUVECs suspension in per plate after 20 min later. Then replaced the serum free media with the complete media included 10% FBS after 6 h. Cells were collected and then extracted mRNA and protein after 48 hours. The sequences for VE-cadherin siRNA were as follows (prepared by Shanghai GenePharma Co., Ltd): sense, 5'-GGAACCAGAUGCACAUUGAUU-3' and anti- sense, 5'-UCAAUGUGCAUCUGGUUCCUU-3'.

The scratch assay

After sevoflurane exposure, the scratch assay was performed immediately. Put out the 6-well plates from the sealed plastic box, sterile yellow pipette tips were used to scratch similar sized wounds on monolayer cells on the super clean bench. Scratched monolayer cells were washed three times by PBS to remove cell fragments and then cultured with serum free media in a incubator, in a humidified atmosphere contains 5% CO₂ and 95% air at 37°C. A microscope was used to observe the migration distance of HUVECs and taken photos. We took photos after just scratched and 12 h later.

Western blot

Poured out the media and washed the cells with PBS three times. HUVECs were lysed in 1×lysis buffer (1 ml RIPA and 10 µl PMSF) and kept on ice for 30 min. Then scraped the cells off the plate and centrifuged 12000 rpm in 4 °C for 20 min then collected the supernatant liquid transferred to new EP tubes for protein quantitation by BCA Protein Assay Kit. The extracted protein was loaded onto 10% SDS-PAGE for electrophoresis with equal amount and then transferred onto a PVDF membrane to transmembrane in 350 mA for 2 h. After transmembrane, the membrane was blocked overnight at 4 °C with 5% skimmed milk. Then the membrane was incubated with primary antibodies for 1 h at 37 °C and overnight at 4 °C later. The membrane was incubated with secondary antibodies at 37 °C for 1 h after washed three times by PBS. Finally, added 1 ml A solution and 1 ml B solution onto the membrane to develop after PBS washed three times again.

Quantitative PCR

RNA was extracted from HUVECs using the Trizol reagent and RNeasy kit. Then these RNA samples were reversely transcribed into single-stranded cDNA by using the first-strand cDNA synthesis kit. These cDNA products were further amplified using qPCR by SYBR Green RT-PCR kit. Sequence specific primers as follows: VE-cadherin, 5'-TCT GTC CCA GAA ATG TCA CG-3' (Forward) and 5'-CAC CGG AGT CAT CAA CTG TG-3' (Reverse); GAPDH, 5'-CCT CTC TGG CAA AGT CCA AG-3' (Forward) and 5'-GGT CAC GCT CCT GGA AGA TA-3' (Reverse). The PCR cycles were as follows: 5 min at 95°C and 10 sec at 95°C including 40 cycles, 15 sec at 65°C, 10 sec at 72°C and a final 1 min at 65°C. Relative expression of qPCR products was normalized with GAPDH mRNA expression.

Flow cytometry

Flow cytometry was used to detect the activity of cells. After sevoflurane exposure, HUVECs were seeded in 6-well plate on the logarithm growth phase then added 2 ml DMEM in each well (4×10⁵ cells/well). After culturing 24 h, HUVECs were fixed by 70% ethanol and were stained. Briefly, cells were mixed with 5

µl propidium iodide (PI) and were incubated in the dark at room temperature for 30 min. The cell activity was detected within 1 h by flow cytometer.

Statistical analysis

Statistical analysis of the data was conducted with SPSS (version 19.0) software and the statistical histograms were derived from GraphPad Prism (version 5) software. Differences between two groups were tested using an unpaired student *t* test. Significant difference was received when $P < 0.05$.

Results

Sevoflurane inhibited HUVECs migration after 2 h exposure

In the scratch assay, the migration distance of cells exposed to 2% sevoflurane for 2 h was significantly shorter than the control group in which cells were exposed to 21% oxygen and 5% CO₂ without sevoflurane. However, the migration distance of HUVECs exposed to 21% oxygen, 5% CO₂ and 2% sevoflurane for 0.5 h and 1 h had no significant difference compared with the control group. The results showed that 2% sevoflurane inhibited the migration function of HUVECs after 2 h exposure (Fig 1A and 1B).

Sevoflurane didn't inhibit the activity of HUVECs

Flow cytometry was used to detect the activity of cells. From the Figure 2, we observed the cell cycle of G1 phase, S phase and G2 phase, and detected cell count in each phase. The flow cytometry results suggested that the cell count of each phase in cell cycle had no significant difference in these two groups. It turned out that the activity of HUVECs exposed to 2% sevoflurane for 2 h had no significant difference with the control group. The result prompted that exposed to 2% sevoflurane for 2 h did not significantly affect the activity of cells (Fig2).

Sevoflurane up-regulated VE-cadherin expression

Quantitative PCR was used to detect the expression of VE-cadherin in HUVECs. The expression of VE-cadherin in the HUVECs exposed to 2% sevoflurane for 2 h had a significant increase compared with the control group, but the HUVECs exposed to 2% sevoflurane for 0.5 h and 1 h had no significant difference compared with the control group. These results suggested that sevoflurane up-regulated VE-cadherin expression to inhibit HUVECs migration function (Fig3).

VE-cadherin siRNA transfection inhibited VE-cadherin expression

Western blot and quantitative PCR results showed that the expression of VE-cadherin in the HUVECs which were transfected with siRNA and not exposed to sevoflurane had a significant decrease compared with the control group, and the expression of VE-cadherin in the HUVECs which were transfected with siRNA and exposed to sevoflurane had no significant difference with the control group (Fig4 and Fig5).

VE-cadherin siRNA transfection promoted HUVECs migration function

In the scratch assay, the migration distance of the HUVECs exposed to 2% sevoflurane for 2 h was significantly decreased compared with the control group. The migration distance of the HUVECs which were transfected with siRNA and exposed to 21% oxygen, 5% CO₂ for 2 h was significantly increased compared with the control group. And the migration distance of the HUVECs which were transfected with siRNA and exposed to 21% oxygen, 5% CO₂ and 2% sevoflurane for 2 h had no significant difference with the control group. These results suggested that VE-cadherin siRNA transfection inhibited the effect of sevoflurane on HUVECs (Fig6A and 6B). Sevoflurane suppressed HUVECs migration may through up-regulating the expression of VE-cadherin.

Discussion

Currently, sevoflurane is widely used in clinical because of its rapid onset of action, quick and complete recovery, good controllability and fewer side effects[19]. But due to its unclear pharmacological mechanism, the effects of sevoflurane on vital organs such as lung, kidney, liver and brain are also inconsistent even though there are many related reports[20~26]. As we all know, the healthy growth of fetus depends on the sufficient blood supply of umbilical vein, therefore, any factor which affects the function of umbilical vein may also affect the normal blood supply of fetus. The effect of sevoflurane exposure on pregnant women and fetus has a lot of research. But in this respect, the effect of sevoflurane exposure on umbilical vein is unknown. Moreover, because the key of the free skin flap transplantation surgery is the survival of the flap, and there are many factors that affect the survival of the flap, among which the most important factors is whether the graft vessel is connected smoothly to ensure adequate blood supply to the flap. The transplanted vessels whether or not carry on the normal functions depend on its normal structure and function. During the transplantation of free skin flap and the anastomosis of blood vessels, this is a time consuming and refined procedure and patients should receive longer time anesthesia. But the potential effect of sevoflurane on the function of vascular endothelium has rarely been reported and whether sevoflurane affects the prognosis of free skin flap transplantation by disturbing the transplanted vessels vascular endothelial cells functions is also unknown.

VE-cadherin as a vascular endothelial cell adhesion molecule plays a necessary role in maintaining vascular endothelium homeostasis[27]. Actually, VE-cadherin is synthesized at the time of endothelial cell differentiation in the embryo, at very early stages[28]. VE-cadherin plays a crucial role in endothelial cell biology, and regulates vascular permeability and vascular integrity by changing its expression[29]. Angiogenesis needs to detach from the vascular wall, invade the underlying tissues and then form tubes. This procedure is complex and involved in endothelial cell proliferation, migration, et al. VE-cadherin plays an important role in vascular remodeling by regulating endothelial cell migration and growth and other specific endothelial cell functions[30]. During the first step of angiogenesis, because VE-cadherin inhibits cell migration from a monolayer, junctions become looser to let cells migration and invade the underlying tissues[31]. When we deeply studied the potential mechanisms of inhibition of cell migration function by sevoflurane, VE-cadherin was selected as one research subject and put forward a hypothesis that VE-cadherin may be involved in the inhibition mechanisms.

In this study, we found that 2% sevoflurane inhibited HUVECs migration function time-dependently. This phenomenon prompts sevoflurane may affect vascular endothelial cell normal functions. Quantitative PCR results showed that HUVECs exposed to 2% sevoflurane for 2 h expressed more VE-cadherin than normal. While HUVECs exposed to 2% sevoflurane for 0.5 h or 1 h respectively did not. The experiments results are consistent with our hypothesis and proposed that the inhibition of cells migration function was time-dependence. We think further whether the inhibition mechanisms of HUVECs migration by sevoflurane also exists concentration-dependence. We will make more efforts to this direction. Moreover, western blot and quantitative PCR results suggested that HUVECs which were transfected with VE-cadherin siRNA and exposed to 2% sevoflurane for 2 h had no significant difference with the control group on the expression of VE-cadherin, and the scratch assay confirmed that the migration distance of the HUVECs which were transfected with VE-cadherin siRNA and exposed to 2% sevoflurane for 2 h had no significant difference with the control group also, which suggested that VE-cadherin involved in the inhibition mechanisms. Our discovery provided that sevoflurane can inhibit vascular endothelial cells normal function, it suggest that sevoflurane may affect the pathologic process repaired by normal vascular endothelial cells functions.

There are obvious shortcomings in the experiment. At first, the experiment was designed in vitro only, no further animal models were designed to verify. Secondly, the molecular mechanisms of inhibition of vascular endothelial cell migration function by sevoflurane require a large number of experiments and further in-depth exploration to verify. Finally, the experimental design was not extended to the clinical field, no clinical trials were carried out to illustrate whether sevoflurane is an influential factor affecting the normal blood supply of fetus and the prognosis of free skin flap transplantation surgery. These deficiencies are the direction we will be going to the next, we will be more in-depth study whether sevoflurane can cause the dysfunction of vascular endothelial cell in the umbilical vein and the transplanted vessels, so then affect the normal function of umbilical vein in pregnant women and the prognosis of free skin flap transplantation surgery.

Conclusions

These results suggested that sevoflurane inhibited the HUVECs migration function by up-regulating VE-cadherin expression, and may have an adverse effect on the normal functions of vascular endothelial cells. So in some operations, such as the free skin flap transplantation surgery, or the non-obstetric surgery during pregnancy, our findings can provide a reference for using sevoflurane in clinical anesthesia.

Abbreviations

HUVECs: human umbilical vein endothelial cells.

MAC: minimal alveolar concentration.

VE-cadherin: vascular endothelial cadherin.

FBS: fetal bovine serum.

DMEM: dulbecco's modified eagle medium.

Declarations

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Author's contributions

TX and YQS conceived the project and analyzed the data, HX and ZFX designed the experiments, YQS performed the experiments and wrote the paper. All authors have read and approved the manuscript, and ensure that this is the case.

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Availability of data and materials

The data and materials used during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was approved by the institutional ethics committees of the international Peace Maternity and Child Health Hospital, School of Medicine, Shanghai Jiao Tong University.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no conflicts of interest concerning this article.

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Figures

Figure 1

A. Photographs under microscope on the HUVECs migration distance were taken when scratched immediately (0 h) and 12 h later (12 h). The “Exposure time” represents that the HUVECs which exposed to 2% sevoflurane for 0.5 h, 1 h and 2 h respectively, and the control group exposed to the same gaseous environment with other three groups but without sevoflurane. B. Statistical histogram of the HUVECs migration distance. X axis represents the exposure time of HUVECs in sevoflurane: 0 h, 0.5 h, 1 h and 2 h respectively. Analysis results showed that the migration distance of HUVECs exposed to 2% sevoflurane for 2 h was significant inhibited compared with the control group (** $p < 0.01$ vs control).

Figure 2

Flow cytometry results showed that the cell viability of HUVECs exposed to 2% sevoflurane for 2 h had no significant difference compared with the control group cells (control). In the figure, the green region on the left represents the G1 phase of the cell cycle; the yellow region in the middle represents the S phase of the cell cycle; the blue region on the right represents the G2 phase of the cell cycle.

Figure 3

Quantitative PCR results showed that the expression of VE-cadherin in the HUVECs exposed to 2% sevoflurane for 2 h had a significant increase compared with the control group (* $p < 0.05$ vs control). And the expression of VE-cadherin in the HUVECs exposed to 2% sevoflurane for 0.5 h and 1 h had no significant difference compared with the control group respectively.

Figure 4

Quantitative PCR results suggested that the expression of VE-cadherin in the HUVECs which were transfected with siRNA and exposed to 2% sevoflurane for 2 h had no significant difference compared with the control group, and the expression of VE-cadherin in the HUVECs which were transfected with siRNA and not exposed to sevoflurane had a significant decrease compared with the control group (* $p < 0.05$ vs control).

Figure 5

Western blot results showed that the change of VE-cadherin expression in protein level. And the expression of protein was normalized by comparison with the level of GAPDH.

Figure 6

Photographs under microscope on the HUVECs migration distance were taken when scratched immediately (0 h) and 12 h later (12 h). The “Control” represents the HUVECs exposed to 21% O₂, 5% CO₂ for 2 h; the “sevo” represents the HUVECs exposed to 21% O₂, 5% CO₂ and 2% sevoflurane for 2 h; the “siRNA” represents the HUVECs transfected with VE-cadherin siRNA and exposed to 21% O₂ and 5% CO₂ for 2 h; and the “sevo+siRNA” represents the HUVECs transfected with VE- cadherin siRNA and exposed to 21% O₂, 5% CO₂ and 2% sevoflurane for 2 h. These comments are also applied to Figure 6B. B. Statistical histogram of the HUVECs migration distance. Analysis results showed that the migration distance of the HUVECs exposed to 2% sevoflurane for 2 h had a significant decrease compared with the control group (**p<0.01 vs control); the migration distance of the HUVECs which were transfected with VE-cadherin siRNA and exposed to 2% sevoflurane for 2 h had no significant difference compared with the control group; and the migration distance of the HUVECs which were transfected with VE-cadherin siRNA and not exposed to sevoflurane had a significant increase compared with the control group (*p<0.05 vs control).