

Agomelatine Attenuates Isoflurane-Induced Inflammation and Damage in Brain Endothelial Cells

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Background and Purpose: Neurotoxicity of anesthetics has been widely observed by clinicians. It is reported that inflammation and oxidative stress are involved in the pathological process. In the present study, we aimed to assess the therapeutic effects of agomelatine against isoflurane-induced inflammation and damage to brain endothelial cells.

Materials and Methods: MTT assay was used to detect cell viability in order to determine the optimized concentration of agomelatine. The bEnd.3 brain endothelial cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μ M) for 24 h. LDH release was evaluated and the ROS levels were checked using DHE staining assay. The expressions of IL-6, IL-8, TNF- α , VEGF, TF, VCAM-1, and ICAM-1 were evaluated using real-time PCR and ELISA. Real-time PCR and Western blot analysis were used to determine the expression level of Egr-1.

Results: The decreased cell viability promoted LDH release and elevated ROS levels induced by isoflurane were significantly reversed by the introduction of agomelatine in a dose-dependent manner. The expression levels of IL-6, IL-8, TNF- α , VEGF, TF, VCAM-1, and ICAM-1 were elevated by stimulation with isoflurane, which were significantly suppressed by the administration of agomelatine. The up-regulation of transcriptional factor Egr-1 induced by isoflurane was down-regulated by agomelatine.

Conclusion: Agomelatine might attenuate isoflurane-induced inflammation and damage via down-regulating Egr-1 in brain endothelial cells.

Keywords: anesthetics, agomelatine, isoflurane, brain endothelial cells, neuroinflammation

Introduction

With the development of modern medicine, more and more fetuses in the third trimester of pregnancy and infants experience the anesthesia process due to clinical examination or surgical operation.¹ The phase from fetuses in the third trimester of pregnancy to infancy is essential for cerebral development, during which a series of developmental processes are involved, including changes in cerebral cortex plasticity, the formation, and maturity of synapses, and neurogenesis. The naïve brain is easily interfered with by external factors during this period.² Multiple animal experiments have verified that apoptosis and long-term cognitive impairment are closely related to the anesthesia experience during the early stages of development.³⁻⁵ Isoflurane, a widely used anesthetic in clinical surgery, is also reported to induce similar neurotoxicity during the developmental phase.⁶ Repeat or long-term anesthesia is proven to impact the normal development of the central

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nervous system.^{7–9} Therefore, it is of great significance to explore the pathological mechanism underlying the neurotoxicity effect of genetic anesthetics.

The mechanism underlying the neurotoxicity effect of genetic anesthetics is relatively complicated, including suppressing the *N*-methyl-D-aspartate (NMDA) receptor, calcium influx induced by the activated gamma-aminobutyric acid receptor (GABA), neuronal apoptosis induced by activated caspase-3, and calcium homeostasis. In addition, oxidative stress can result from the damage to morphology, integrity, and function of mitochondria induced by genetic anesthetics, which finally induces the apoptosis of neurons.¹⁰ Other than neurons, the apoptosis of brain endothelial cells, which are the main components of the blood-brain-barrier (BBB), can also be induced by the administration of isoflurane.^{11,12} Recently, it is reported by several studies that a disrupted BBB or aggravated brain edema could be induced in the *in vitro* BBB model, the rats post transient focal cerebral ischemia^{13,14} and a mouse model of traumatic brain injury¹⁵ by the introduction of isoflurane.¹² The disruption of tight junctions¹⁶ and excessive oxidative stress are the two mechanisms proposed that are underlying the BBB opening after the administration of isoflurane.¹⁷ In animal models, an inflammation-mediated BBB opening has been observed after performing surgeries on animals under general anesthesia with isoflurane.^{18,19} Taken together, to protect the brain from the side effects of isoflurane, suppressing the induced oxidative stress and inflammation in the brain endothelial cells might be effective.

Agomelatine, the molecular structure of which is shown in Figure 1, is a novel synthetic chemical reagent with anti-depression effects, the mechanism of which includes mediating the releasing of neurotransmitters by regulating the protein phosphorylation cascades and impacting the neuroplasticity of the brain such as at the suprachiasmatic nucleus.^{20,21} As an agonist of the melatonin receptor and selective inhibitor for the serotonin 2C receptor, agomelatine showed a promising anti-depression effect reported through several pre-clinical and clinical studies.²² Recently, the neuroprotective effect of agomelatine has been widely reported both *in vitro* and *in vivo*.^{23,24} In the present study, the protective effect of agomelatine against damaged brain endothelial cells by isoflurane was investigated to seek the potential therapeutic method for the treatment of clinical side effects in the brain induced by isoflurane.

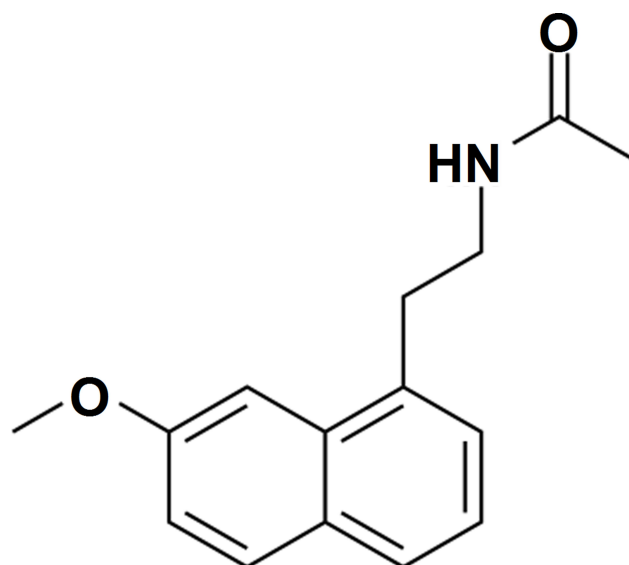


Figure 1 Molecular structure of agomelatine.

Materials and Methods

Cell Culture and Treatment

BEnd.3 brain endothelial cells were purchased from the Chinese Academy of Science (CAS, Shanghai, China) and cultured in the DMEM cultural medium containing 10% FBS and 1% penicillin–streptomycin at 37°C in a 5% CO₂ incubator. The BEnd.3 cells were maintained at health status and seeded on the 6-well plate at the density of 5×10^5 cells per well and allowed to grow for 48 h up to 80–90% confluence for the treatment experiment. To measure the effects of agomelatine on the cell viability of bEnd.3 brain endothelial cells, cells were treated with agomelatine at the concentrations of 0.1, 0.5, 1, 5, 10, 50, and 100 μ M for 24 h. To assess the protective effects of agomelatine against isoflurane, cells were treated with 2% isoflurane in the presence or absence of 5 and 10 μ M agomelatine for 24 h, which were optimized as the non-toxic doses. For each cell-based assay, the mean of 3–5 replicates was obtained, and the final data were presented as fold change.

MTT Assay

Cell viability of treated bEnd.3 brain endothelial cells was evaluated using the MTT assay. Briefly, MTT reagent in serum-free DMEM medium at a final concentration of 5 mg/mL was administrated into each well and incubated at 37°C for approximately 4 h, followed by removing the medium and dissolving in 200 μ L of 0.1 N acidic isopropyl alcohol. The calorimetric measurement of MTT reduction was recorded at

570 nm on a microplate reader (BioTek, Winooski, USA). The background absorbance value of the blank was subtracted from all test samples. The measured value of control cells incubated with DMEM was taken as 100% viability, and the data were presented as fold change to control cells.

LDH Releasing Assay

The collected medium of culture cells was mixed with 50 μ L of reaction solution using a commercial LDH detecting kit (Sigma-Aldrich, St. Louis, USA), followed by incubation at room temperature in the dark for 30 minutes. Subsequently, 50 μ L stop solution was added into each well and incubated for another 30 minutes. A microplate reader (Thermo Fisher Scientific, Waltham, USA) was used to read the absorbance at 492 nm.

Dihydroethidium (DHE) Staining

The production of superoxide from the endothelial cells was evaluated using the DHE assay. Briefly, the cells were stained with DHE (4 μ M) for 30 minutes and the cellular images were captured under fluorescence microscopy (Nikon, Shinagawa, Japan). The detailed procedure was previously described.²⁵

Quantitative Real-Time PCR (qRT-PCR)

The TRIzol reagent (Thermo Fisher, Waltham, USA) was used to isolate total RNA from endothelial cells. Subsequently, 2 μ g of total RNA with a Primescript material kit (Thermo Fisher, Waltham, USA) was used to synthesize cDNA. Real-time PCR amplification was performed using a TransStart Tip Green qPCR SuperMix kit (Genscript, Piscataway, USA). The following primers were used in this study: mouse IL-6 (Forward: 5'-CCAATTTCCAATGCTCTCCT-3'; Reverse: 5'-ACCACAGTGAGGAATGTCCA-3');

IL-8 (Forward: 5'-GGGTGGGGAGTTCGTGTAGA-3'; Reverse: 5'-CTACTACACAGGGATCAGGGC-3');

TNF- α (Forward: 5'-TTCCGAATTCAGTGGAGCC TCGAA-3'; Reverse: 5'-TGCACCTCAGGGAAGA ATCTGGAA-3');

VEGF (Forward: 5'-GAGCAGAAGTCCCATGA AGTGA-3'; Reverse: 5'-CACAGGACGGCTTGAAGA TGT-3');

VCAM-1 (Forward: 5'-GCCCACTAAACGCGAAG GT-3'; Reverse: 5'-ATGGTCAGAACGGACTTG GAC-3');

ICAM-1 (Forward: 5'-CGCTGTGCTTTGAGAACT GTG-3'; Reverse: 5'-ATACACGGTGATGGTAG CCGA-3');

TF (Forward: 5'-GACGAGATCGTGAAGGATGT -3'; Reverse: 5'-CAGATAGTGACAGGAGGATGAT-3');

Egr-1 (Forward: 5'-TTGTGGCCTGAACCCCTTTT -3'; Reverse: 5'-AGATGGGACTGCTGTCGTTG-3');

GAPDH (Forward: 5'-ACTCCCACTCTTCCACCTTC -3'; Reverse: 5'-TCTTGCTCAGTGTCTTGC-3');

Western Blot Analysis

Cells were collected and lysed in RIPA lysis buffer (Thermo Fisher, Waltham, USA). Fifteen percent SDS-PAGE was used to separate the proteins. Subsequently, the isolated proteins were transferred to the PVDF membranes (Thermo Fisher, Waltham, USA) by semi-dry transfer. BSA solution (5–10%) was added and hatched for 1–2 h. Then, the membranes were incubated with primary antibodies against Egr-1 (#ab194357, Abcam, USA) or β -actin (#ab8226, Abcam, USA) at 25°C for 2 h. After being washed, horseradish peroxidase (HRP)-conjugated secondary antibody (1:3000, Abcam) was used to incubate with the membranes at 25 °C. One to two hours later, blots were incubated with the ECL reagents (Amersham, Little Chalfont, UK) and exposed under Amersham Imager 600 (GE, Boston, USA).

ELISA Assay

The released levels of IL-6 (#M6000B), IL-8 (#D8000C), TNF- α (#MTA00B), VEGF (#MMV00), TF (#DY2339), VCAM-1 (#DY643), and ICAM-1 (#MIC100) were measured using commercial ELISA kits (R&D Systems, Minneapolis, USA). Briefly, 100 μ L different dilutions (from 50 to 5000 pg/mL) of the standard sample and test samples were added together with PBS buffer to the reaction plate and incubated at 37 °C for 30 minutes. After washing with PBS, 100 μ L of the test solution was added to each well and incubated at 37°C for 2 h. Thereafter, the plates were washed with PBS prior to the addition of 100 μ L horseradish peroxidase (HRP)-labeled secondary antibody and incubated at 37 °C for 30 minutes. Then, 50 μ L developer A and 50 μ L developer B were added. The plates were kept in the dark for 15 minutes. Finally, 50 μ L stop solution was added to the plate to stop the reaction. The optical density of the plate was read at 450 nm using an ELISA reader (EXL808; BioTek Instruments), and the concentration of the test sample was calculated.

Statistical Analysis

Mean \pm standard deviation (S.D.) was displayed to show the data. GraphPad was used to analyze the data. Analysis of variance (ANOVA) followed by Tukey's HSD post hoc test was used for the contrast among different groups. $P < 0.05$ was regarded as a statistically significant difference between the groups.

Results

The Determination of the Optimized Agomelatine Concentration

To screen the optimized concentration of agomelatine used in the present study, MTT assay was used to evaluate the cell viability of endothelial cells following treatment with agomelatine at the concentrations of 0.1, 0.5, 1, 5, 10, 50, 100 μM for 24 h. As shown in Figure 2, no significant decrease in cell viability was observed as the concentration of agomelatine increased from 0.1 to 10 μM . However, a significant decrease in cell viability was observed when the concentration of agomelatine was increased to 50 or 100 μM . Therefore, 5 and 10 μM were chosen as the optimized concentrations used in the present study.

Agomelatine-Ameliorated Isoflurane-Induced Reduction of Cell Viability and the Release of LDH in bEnd.3 Brain Endothelial Cells

The bEnd.3 brain endothelial cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μM) for 24 h. Figure 3A shows the morphology of cells from each group. As shown in Figure 3B, the cell viability

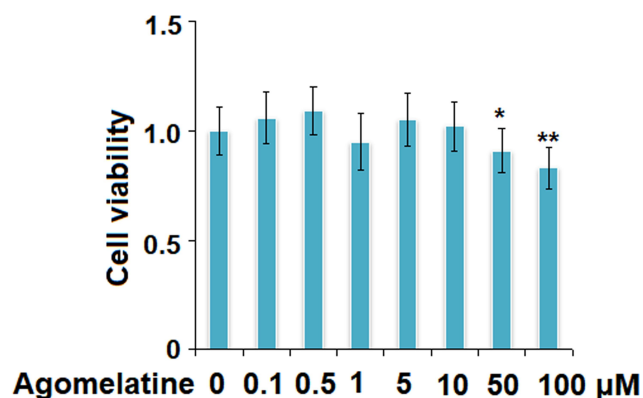


Figure 2 The effects of agomelatine in cell viability of bEnd.3 brain endothelial cells. Cells were treated with agomelatine at the concentrations of 0.1, 0.5, 1, 5, 10, 50, 100 μM for 24 h. Cell viability was measured using MTT assay (N=3, *, **, $P < 0.05$, 0.01 vs control group).

was significantly inhibited by the stimulation with isoflurane and was greatly rescued by the introduction of agomelatine in a dose-dependent manner. Figure 3C shows the LDH production by the treated endothelial cells. We found that the increased LDH release induced by 2% isoflurane was significantly suppressed by the introduction of agomelatine.

The Oxidative Stress and Production of Inflammatory Factors Induced by Isoflurane Were Alleviated by Agomelatine

The ROS levels and concentrations of inflammatory factors were determined after the cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μM) for 24 h. As shown in Figure 4, the ROS levels were significantly elevated by the stimulation with isoflurane, which was greatly inhibited by the administration of agomelatine in a dose-dependent manner. Figure 5A shows the gene expression level of inflammatory factors released by the treated endothelial cells. We found that the up-regulated IL-6, IL-8, and TNF- α induced by isoflurane were significantly down-regulated by the introduction of agomelatine. The inhibition of agomelatine on these cytokines was confirmed at protein level. As shown in Figure 5B, exposure to 2% isoflurane induced high levels of IL-6, IL-8, and TNF- α release in the media, but the presence of 5 and 10 μM agomelatine dose-responsively reduced cellular IL-6, IL-8, and TNF- α release.

The Dysfunction of Endothelial Cells Was Alleviated by Agomelatine

To evaluate the effects of agomelatine on the impaired function of endothelial cells induced by isoflurane, the expression levels of VEGF and tissue factor (TF) were determined after the cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μM) for 24 h. As shown in Figure 6A, the expression levels of VEGF and TF were significantly increased by the stimulation with isoflurane, which were greatly suppressed by the introduction of agomelatine in a dose-dependent manner. The inhibitive effect of agomelatine on VEGF and TF mRNA was confirmed at protein level. As shown in Figure 6B, the exposure to 2% isoflurane induced high production of VEGF and TF in the media, but the addition of 5 and 10 μM agomelatine dose-responsively inhibited VEGF and TF release.

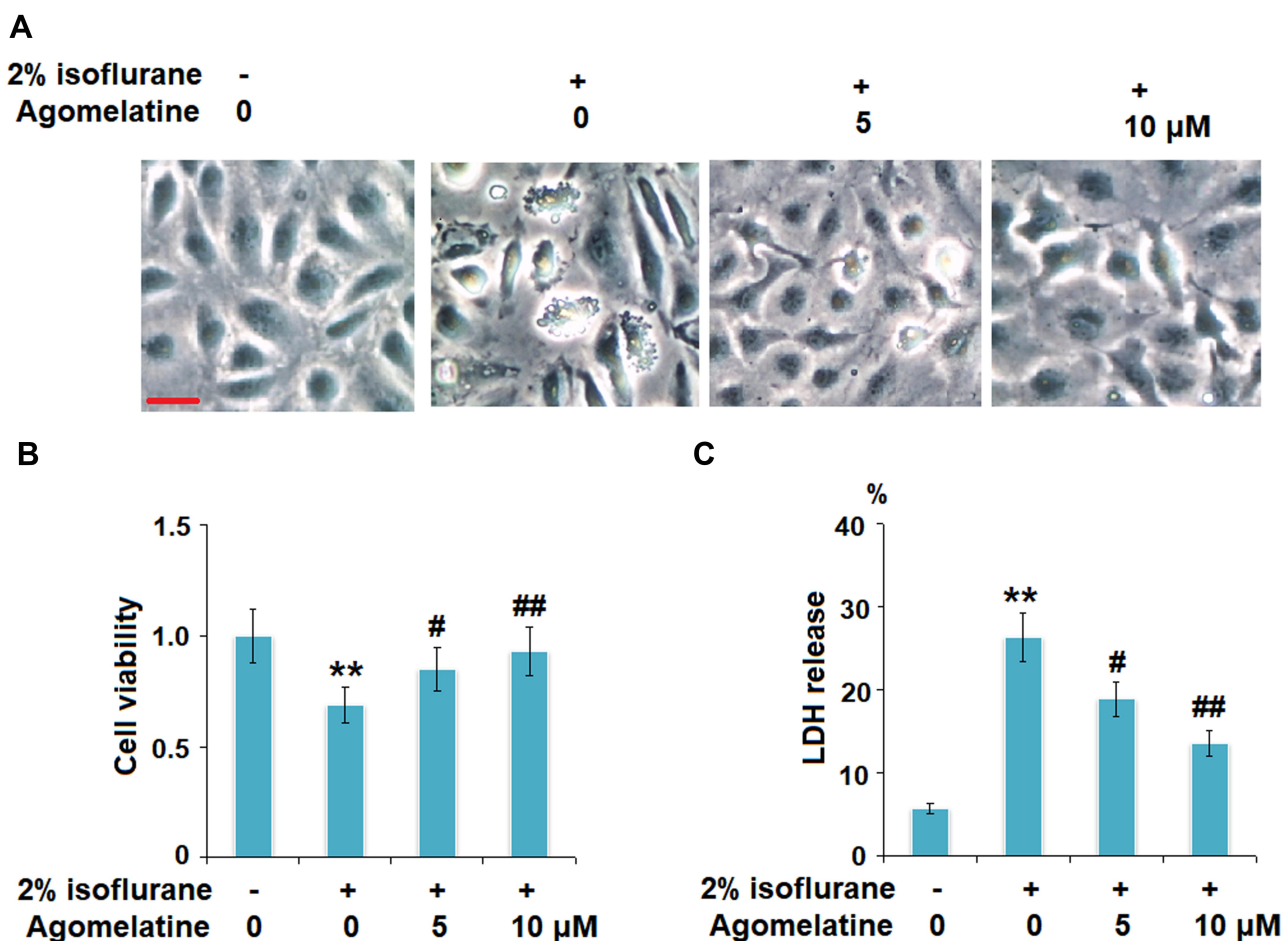


Figure 3 Agomelatine-ameliorated isoflurane-induced reduction of cell viability and release of lactate dehydrogenase (LDH) in bEnd.3 brain endothelial cells. Cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μ M) for 24 h. (A) Morphology of bEnd.3 brain endothelial cells; Scale bar, 100 μ m. (B) Cell viability was measured by MTT assay. (C) LDH release was measured by a commercial kit (N=3, **, P<0.01 vs control group; #, ##, P<0.05, 0.01 vs isoflurane group).

The Up-Regulation of VCAM-1, ICAM-1, and Egr-1 Induced by Isoflurane Was Reversed by Agomelatine

To evaluate the effects of agomelatine on adhesion function of endothelial cells, qRT-PCR and ELISA were used to determine the expression levels of VCAM-1 and ICAM-1 after cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μ M) for 24 h. As shown in Figure 7A, the up-regulated gene of VCAM-1 and ICAM-1 stimulated by isoflurane was significantly down-regulated by the administration of agomelatine in a dose-dependent manner. The suppression of agomelatine on VCAM-1 and ICAM-1 mRNA was verified at protein level. As shown in Figure 7B, the exposure to 2% isoflurane induced high secretions of VCAM-1 and ICAM-1 in the media, but the addition of 5 and 10 μ M agomelatine dose-responsively inhibited VCAM-1 and ICAM-1 release.

Meanwhile, qRT-PCR and Western blot were used to evaluate the expression level of transcriptional factor Egr-1 after cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μ M) for 24 h. As shown in Figure 8A and B, Egr-1 was significantly up-regulated by the stimulation with isoflurane but was greatly down-regulated by the introduction of agomelatine in a dose-dependent manner.

Discussion

The BBB is an important biological structure that separates blood circulation from the brain tissue, and it mainly consists of tight junction endothelial cells, basilemma, astrocytes, and pericytes. The function of the BBB is mainly maintained by the tight junctions constructed by endothelial cells and impacted by the regulation of astrocytes, pericytes, microglia, and neurons. The integrity of the BBB is of great significance in maintaining the

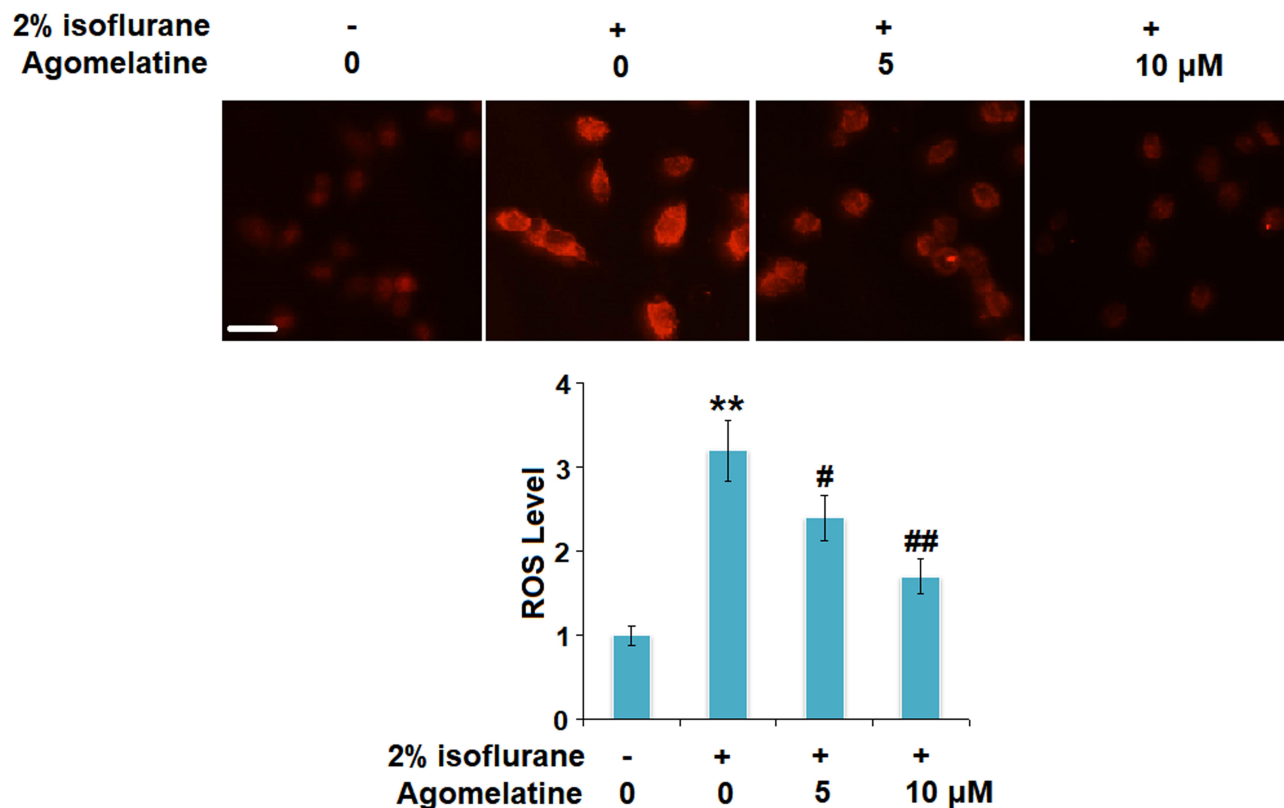


Figure 4 Agomelatine prevented isoflurane-induced oxidative stress in bEnd.3 brain endothelial cells. Cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μM) for 24 h. Intracellular reactive oxygen species (ROS) was measured by dihydroethidium (DHE) staining. Scale bar, 100 μm (N=3, **, P<0.01 vs control group; #, ##, P<0.05, 0.01 vs isoflurane group).

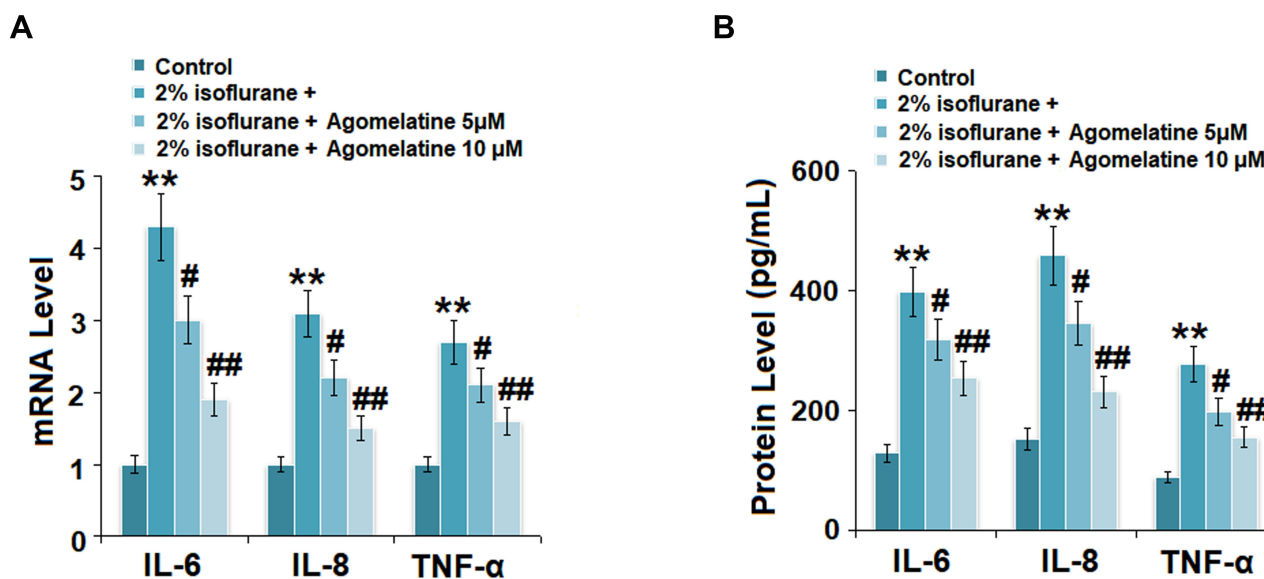


Figure 5 Agomelatine inhibited isoflurane-induced expression and production of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α. Cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μM) for 24 h. (A) mRNA of IL-6, IL-8, and TNF-α (N=5). (B) Secretions of IL-6, IL-8, and TNF-α as measured by ELISA (N=5, **, P<0.01 vs control group; #, ##, P<0.05, 0.01 vs isoflurane group).

stability of the microenvironment in brain tissue and protecting the normal function of the central nervous system.²⁷ Pathogenic microorganisms and other

macromolecular substances in the blood circulation can be blocked by the BBB from entering the brain tissue. The destruction of the BBB induces serious toxic effects,

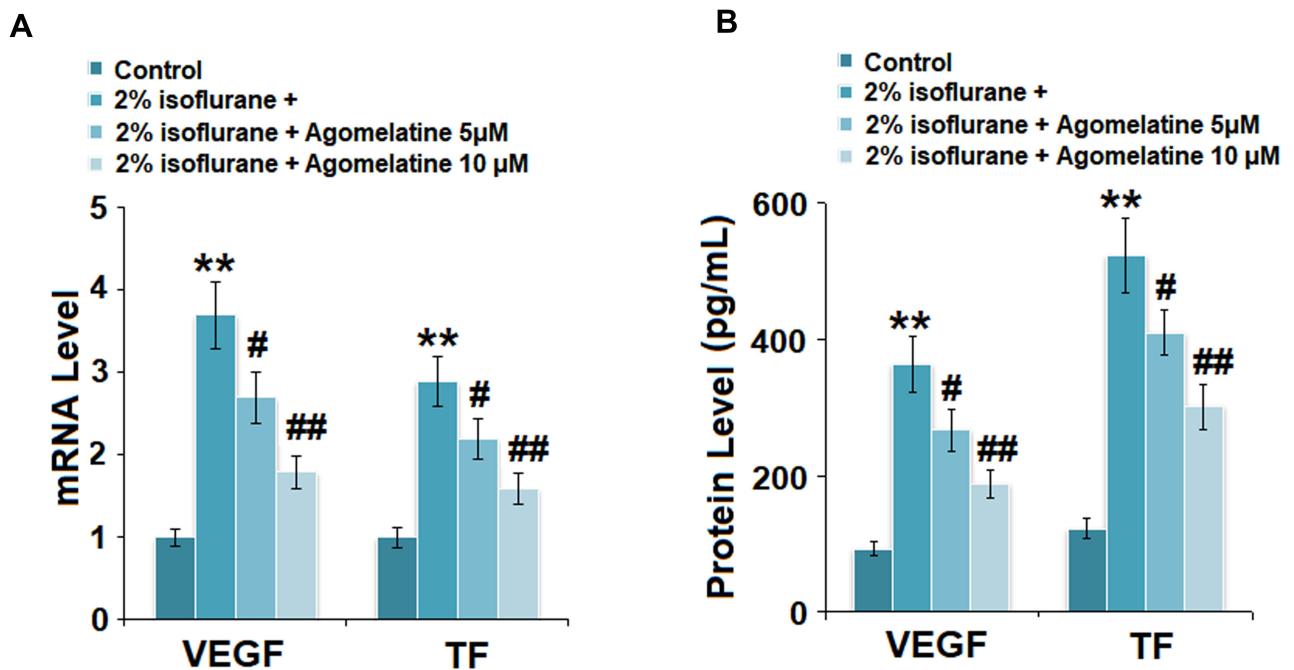


Figure 6 Agomelatine suppressed isoflurane-induced expression of VEGF and tissue factor (TF) in bEnd.3 brain endothelial cells. Cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μM) for 24 h. (A) mRNA of VEGF and TF as measured by real-time PCR (N=5). (B) Protein levels of VEGF and TF as measured by ELISA (N=5, **, P<0.01 vs control group; #, ##, P<0.05, 0.01 vs isoflurane group).

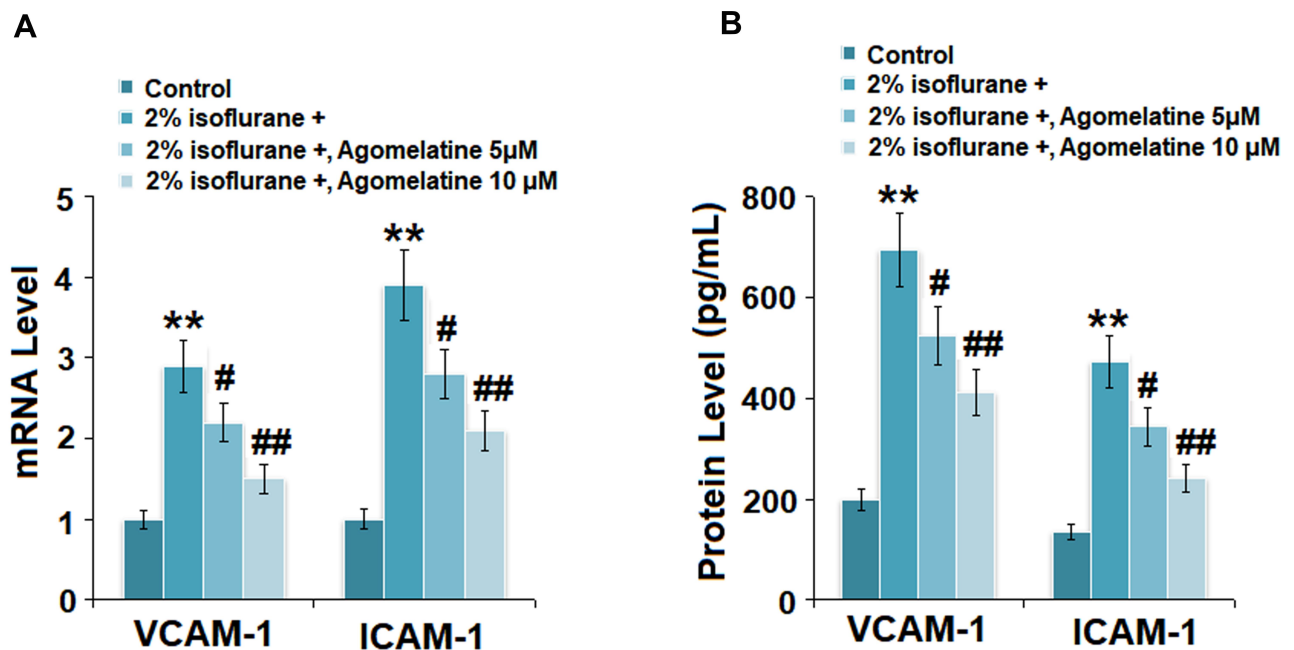


Figure 7 Agomelatine reduced isoflurane-induced expression of cell adhesion molecules VCAM-1 and ICAM-1. Cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μM) for 24 h. (A) mRNA levels of VCAM-1 and ICAM-1 as measured by real-time PCR (N=5). (B) Protein levels of VCAM-1 and ICAM-1 as measured by ELISA (N=5, **, P<0.01 vs control group; #, ##, P<0.05, 0.01 vs isoflurane group).

including vasogenic or cellular cerebral edema, metabolic imbalance of brain tissue, production of excitotoxicity, the infiltration of peripheral macrophages and lymphocytes, inducing inflammation, and inhibiting the repairment of

neurons.²⁸ BBB destruction by the application of anesthesia during clinical surgery has been widely reported. Zhu reported that the postoperative cognitive dysfunction and destruction of the BBB induced by isoflurane could be

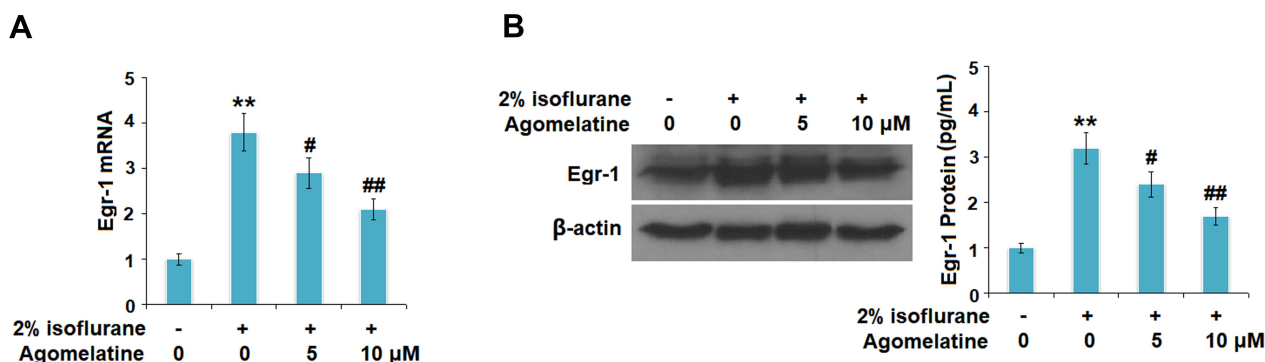


Figure 8 Agomelatine reduced isoflurane-induced expression of the transcriptional factor Egr-1. Cells were treated with 2% isoflurane in the presence or absence of agomelatine (5, 10 μM) for 24 h. (A) mRNA of Egr-1 as measured by real-time PCR (N=5). (B) Protein of Egr-1 as measured by Western blot analysis (N=5, **, P<0.01 vs control group; #, ##, P<0.05, 0.01 vs isoflurane group).

worsened by the inflammation caused by peripheral immune cells that cross the injured mouse BBB.²⁹ Dittmar reported that the apoptosis of endothelial cells could be induced by the introduction of isoflurane in an in-vitro BBB model.¹¹ In the present study, bEnd.3 brain endothelial cells were used to evaluate the toxicity of isoflurane, which was verified by the decreased cell viability, promoted LDH release, elevated ROS levels, excessive production of inflammatory factors, and up-regulated cell adhesion molecules. By the introduction of agomelatine, we found that the toxicity of isoflurane against the endothelial cells was significantly reversed, which was verified by the increased cell viability, decreased LDH release, suppressed ROS levels, inhibited the production of inflammatory factors, and down-regulated cell adhesion molecules.

Among these biological changes, the inhibitory effect of agomelatine against ROS levels deserved to be paid close attention to, as it was an important biomarker of oxidative stress. Oxidative stress is reported to be one of the main inducers of apoptosis of endothelial cells, especially in the pathological process of brain injuries, such as brain ischemia-reperfusion injury. Chen reported that the apoptosis of vascular endothelial cells could be induced by a high dosage of glucose through the activation of NADPH oxidase-driven oxidative stress.³⁰ Free fatty acids are also reported to induce the apoptosis of human brain vascular endothelial cells by inducing oxidative stress through regulating the Akt pathway.³¹ In our future work, further investigations will be explored on the inhibitory effects of agomelatine against oxidative stress, including the level of reduced GSH and the underlying molecular signaling pathways, such as the Keap1/Nrf2 signaling pathway. In addition, due to the toxic effects of

isoflurane on endothelial cells, especially the activation of oxidative stress, the application of anesthetics during surgeries for brain injury-induced diseases, such as brain ischemia-reperfusion injury, should be given close attention. The destruction of the BBB can be accentuated by the usage of anesthetics, which further aggravates brain injury.

Egr-1 is a zinc-finger like transcription factor, which belongs to the family of immediate early genes.³² It is reported that Egr-1 is significantly up-regulated in the pathological process of lung ischemia/reperfusion injury, acute injury of the femoral artery, and myocardium ischemia/reperfusion injury. The excessive production of pro-inflammatory factors and thrombogenic molecules, such as PAI-1, TF, ICAM, VCAM, and MMPs, can be induced by Egr-1.^{33,34} In the present study, we found that the expression level of Egr-1 could be elevated by the stimulation with isoflurane, which was significantly suppressed by the administration of agomelatine, indicating that Egr-1 might be a key factor that mediates the effects of agomelatine against the injuries by isoflurane on the endothelial cells.

In prenatally induced autism animals, agomelatine therapy has shown a protective effect on the BBB leakage, oxidative stress, and inflammation on the brain.³⁵ In patients with major depression disorder, administration of agomelatine reduces serum CRP.³⁶ Circulated CRP level is a sensitive risk factor of cardiovascular diseases.³⁷ Melatonin MT1 and MT2 receptors are G protein-coupled receptors expressed in various parts of the CNS and peripheral blood vessels.³⁸ In the vasculature, melatonin promotes vasoconstriction through MT1 receptor activation, and also enhances vasodilatation through MT2 receptor activation, this dual regulation indicates that MT1 and MT2 receptor activation play an important role in vascular function.³⁹ A recent study finds that the injection of agomelatine

inhibits myocardial mitochondrial permeability transition pore opening and promotes myocardial ischemia healing. Therefore, agomelatine-mediated melatonin receptor activation could have potential modulation in cardiovascular diseases.

The limitations of the current study have to be mentioned. In this study, we tested the protective role of agomelatine in isoflurane-mediated endothelial injury; however, very little is known about how agomelatine acts to suppress inflammation and protect endothelial cells from neurotoxic agents. The identification of transcriptional factor Egr-1 indicates that this could be one of the key mechanisms involved in the action of agomelatine in endothelial protection. Currently, we are still not sure how agomelatine-ameliorated endothelial Egr-1 expression is associated with its activation of melatonin receptor activation. Further investigations should be performed to verify the conclusion in our future work, such as involving the activator of Egr-1 or up-regulating the expression level of Egr-1 using transgenic technology. Ultimately, the in vivo test of agomelatine's effect in appropriate animal models is required to understand its potential role in drug-caused neurotoxicity.

Collectively, our data indicate that agomelatine might attenuate Isoflurane-induced inflammation and damage in brain endothelial cells by suppressing inflammation and oxidative stress. Our study implies the anti-depression drug agomelatine could have a protective role against anesthetics-induced endothelial injury.

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Disclosure

The authors report no conflicts of interest for this work.

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