Sevoflurane Inhibits Proliferation and Invasion of Human Ovarian Cancer Cells by Regulating JNK and p38 MAPK Signaling Pathway

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Aim: Sevoflurane is a halogen inhaled anesthetic, and we aimed to probe the effect of sevoflurane on proliferation and invasion of human ovarian cancer (OC) and its mechanism.

Methods: OC cell lines were divided into 4 groups including control, sevoflurane low concentration (1.7%), medium concentration (3.4%) and high concentration (5.1%) groups. Flow cytometry and MTT assay were, respectively, employed to detect the cell apoptosis and proliferation. Transwell assay was applied to measure the cell migration and invasion viability. The gene and protein expressions were assessed using qRT-PCR and Western blot. The expressions of MAPK signaling pathway-related proteins were evaluated by Western blot. The p38 and JNK inhibitors were, respectively, added into the high concentration group to analyze the relationship between sevoflurane and modulating mitogen-activated protein kinase (MAPK) pathway in OC. Nude mice models were constructed to explore the effect of sevoflurane on OC tumor growth in vivo.

Results: Sevoflurane inhibited OC proliferation in vitro and in vivo. It could also promote OC cell apoptosis in a dose-dependent manner. Sevoflurane suppressed the OC cell migration and invasion, and these effects were positively correlated with the dose of sevoflurane. Moreover, sevoflurane treatment inhibited the expressions of PCNA, Twist, cleaved-caspase-3/caspase-3, MMP-2 and MMP-9. In addition, sevoflurane repressed the phosphorylation of JNK and p38 MAPK. When the MAPK pathway was interdicted, the cell proliferation, apoptosis, migration and invasion activity were recovered after sevoflurane treatment.

Conclusion: Sevoflurane affected cell biological activities in OC by regulating JNK and p38 MAPK signaling pathway.

Keywords: ovarian cancer, sevoflurane, proliferation, migration and invasion, MAPK signaling pathway

Introduction

Ovarian cancer (OC), which has an obscure onset and lacks early clinical symptoms, is common in perimenopausal women. The incidence of OC is the third highest among all gynecological tumors, after cervical cancer and endometrial cancer, but its mortality rate is the first. Worldwide, there were 300,000 new OC cases in 2018 and 200,000 deaths from OC. In China, 52,100 new cases of OC and 22,500 deaths were reported in 2016. Unlike other tumors, OC tumors tend to invade and metastasize within the peritoneum, rarely through blood vessels or lymph nodes. Currently, the clinical treatment strategies for OC are very limited,
mainly including surgery, chemotherapy and chemotherapeutic drugs. Many OC cases were found at the advanced stage of extensive metastasis, more than 75% of the women at the time of diagnosis is to late stage (III or IV). Half of the OC patients relapsed within 16 months, and the 5-year overall survival rate was less than 50%, while the 5-year survival rate for early-stage OC was also very low. The main reasons for the poor diagnosis and prognosis of OC are that OC usually presents asymptomatic characteristics in the early stage and easy to develop resistance to traditional platinum chemotherapy drugs.

For the past three decades, the standard treatment for OC has been tumor cell ablation, supplemented by taxoid-based and platinum-based chemotherapy. Even after standard treatment, most patients showed resistance to chemotherapy and relapsed. Chemotherapy-resistant patients were often treated with second-line or higher chemotherapy agents such as topotecan, gemcitabine, liposomal doxorubicin, and etoposide. Unfortunately, the progression-free survival of these patients was only 2–4 months, and the overall survival was 10–14 months. Therefore, finding more effective treatment or drugs to inhibit OC was helpful to reduce the mortality of OC and improved the survival status of patients. Sevoflurane is a commonly used inhalation anesthetic drug. Previous studies have shown that sevoflurane could play a role in a variety of tumors, such as colon cancer, breast cancer, lung cancer and cervical cancer. These reports suggested that sevoflurane had potential clinical application value in malignant tumor, but its effect on malignant behavior of OC cells and mechanism had not been completely clarified.

In the current study, we explored the effect of sevoflurane treatment on OC cells and its functional mechanism. We investigated that sevoflurane inhibited the OC cell lines (SKOV3 and OVCAR3) proliferation in vitro and in vivo. And the OC cell apoptosis was enhanced after sevoflurane treatment. Sevoflurane could also decrease the OC cells migration and invasion viability. All of these effects of sevoflurane on OC cell lines were positively correlated with the concentration of sevoflurane. In addition, we demonstrated that sevoflurane treatment effected on OC cells via modulatingmitogen-activated protein kinase (MAPK) signaling pathway. These findings suggested that sevoflurane might be a potential and novel agent in OC therapy.

Materials and Methods

Cell Culture

Human OC cell lines OVCAR3 and SKOV3, which were provided by Shanghai Cell Bank, Chinese Academy of Sciences (Shanghai, China), were used in this study. OC cell lines SKOV3 and OVCAR3 were cultured into DMEM medium with 10% fetal bovine serum (FBS, Invitrogen Carlsbad, CA, USA), and the culture environment was 37°C and 5% CO₂.

Protocol of OC Cells Exposure to Sevoflurane

Sevoflurane treatment of cells referred to previous studies. In brief, cell culture plates were placed in a sterile and closed container with inlet and outlet connectors. An anesthesia machine (GE Healthcare Life Sciences, Chalfont, UK) was connected to the inlet port, and attached to a sevoflurane vaporizer (Sevorane; Abbott, Abbot Park, IL), which was used to supply sevoflurane gas (mixed with 95% O₂/5% CO₂) into the container. The concentrations of sevoflurane in the container were monitored by a gas monitor (PM8060, Drager, Lübeck, Germany), which was connected to the outlet port of container. The cell lines were exposed to different concentrations (1.7% v/v, 3.4% v/v or 5.1% v/v) of sevoflurane for 6 h, and, respectively, divided into 4 groups: Control group, high sevoflurane concentration group (5.1% group), medium sevoflurane concentration group (3.4% group) and low sevoflurane concentration group (1.7% group). The cells of the control group only treated by a mixture of 95% O₂ + 5% CO₂. Then, the cells were cultured in normal conditions for 24 h before further analyses.

MAPK Signaling Pathway Inhibitor Treatment

To block MAPK signaling pathway, the SKOV3 cell lines were incubated with p38 inhibitor SB203580 (20 μM, ab120162, Abcam, Cambridge, MA, USA) or JNK inhibitor SP600125 (10 μM, ab120065, Abcam) according to the manufacturer’s instructions. Briefly, the confluent SKOV3 cell lines were pre-incubated for 8 h with SB203580 or SP600125 prior to exposure to 5.1% sevoflurane for 4 h. Then, the cells were divided into 4 groups: control group, 5.1% group, 5.1% + p38 inhibitor group (5.1% + SB203580 group) and 5.1% + JNK inhibitor group (5.1% + SP600125 group). The cell proliferation, apoptosis, migrations and invasion were detected at
24 h post-sevoflurane treatment. Each treatment was performed in triplicate.

3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

The cell proliferation activity was measured using MTT assay. After sevoflurane treatment for 24 h, cells were seeded into 96-well plates with a dose of 5000 cells for one well. Briefly, 10 μL of MTT solution (5 mg/mL, Thermo Fisher, Wilmington, DE, USA) was used to incubate cells for 4 h, and then 200 μL of dimethylsulfoxide (DMSO, Thermo Fisher) was added into each well for 10 min. Absorbance was evaluated at 490 nm using a microporous plate spectrophotometer (Thermo Labsystems, Vantaa, Finland). MTT assay was repeated 3 times.

Cell Apoptosis

The cell apoptosis was detected using flow cytometry. After sevoflurane treatment for 24 h, cell lines were digested and collected by trypsinase (Invitrogen). The cells were washed with precooled PBS (Invitrogen) for 2 times, and 1 × 10^6 cells/mL cell suspension was prepared. Cell suspensions (100 μL/tube) were stained by propidium iodide (Nanjing KeyGen Biotech, Nanjing, China) and fluorescein isothiocyanate-conjugated Annexin V (Nanjing KeyGen Biotech) according to the instructions. The cells were gently mixed and placed in dark for 15 mins. The results were determined by flow cytometry (BD Biosciences, NJ, USA) within 1 h. Flow cytometry assay was repeated 3 times.

Transwell Assay

The cell migration and invasion viability were both measured by transwell assay. For migration viability, FBS-free DMEM medium was used to resuspend cells. The concentration of cells was adjusted to 1 × 10^6 cells/mL. 200 μL cell suspension was added to each chamber. A total of 600 μL of DMEM medium (10% FBS) was added to each pore in the lower chamber. After 24 h of incubation, the cells were removed from the chamber and wiped off with cotton swabs. The cells were stained with 0.1% crystal violet for 20 mins. 200× field microscope images were taken (5 fields/group). Migration inhibition rate = (1 − number of migrating cells in experimental group/number of migrating cells in control group) × 100%. All experiments were repeated 3 times.

RNA Extraction and Real-Time Quantitative Polymerase Chain Reaction (qRT-PCR)

Trizol kits (Invitrogen) were used to extract the total RNA from human OC cell lines, the RNA reverse transcription with RT MasterMix PrimeScript™ kit for cDNA (TaKaRa, Dalian, China). qRT-PCR was proceeded with SYBR Premix Ex Taq™ II kit (TaKaRa) using cDNA as a template according to the manufacturer’s instruction, GAPDH was used as the internal control. Relative quantitative method was used to determine the results and 2^−△△Ct was calculated. The experiment was repeated for three times. The primer sequences used in this study were as follows: PCNA forward primers 5′-GCCTGAC AAATGCTTGCT-3′, reverse primers 5′-GCCGGGAAGGA

Western Blot

Radio Immunoprecipitation Assay (RIPA) lysis buffer, provided by Sigma (St. Louis, MO, USA), was used to collect protein lysates. BCA method was applied to quantify the total protein concentration. Proteins were added to SDS polyacrylamide gel electrophoresis (PAGE), and electrotransferred to polyvinylidene fluoride (PVDF) membrane after electrophoresis separation. Blocking buffer (5% bovine serum albumin, BSA) was used to block the membranes at room temperature. After blocking, primary antibodies were used to incubate the membranes overnight at 4°C. Primary antibodies (Abcam, Cambridge, MA, USA) were used at the following dilutions: anti-PCNA (1:1000, ab18197), anti-cleaved-caspase-3 (1:1000, ab2302), anti-caspase-3 (1:500, ab13847), anti-Twist (1:2000, ab175430), anti-MMP-2 (1:1000, ab37150), anti-MMP-9 (1:1000, ab73734), anti-phosphorylation-p38 (p-p38) (1:1000, ab4822), anti-p38 (1:1000, ab31828), anti-p-JNK (1:1000, ab124956), anti-JNK (1:1000, ab179461), anti-p-ERK (1:1000, ab65142), anti-ERK (1:10,000, ab32537), anti-GAPDH (1:500, ab8245). After washing the membranes Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 4 times, the secondary antibody was added and incubated at 37°C for 30 min. GAPDH served as the loading control. All experiments were repeated 3 times.
GGAAAG-3'; caspase-3 forward primers 5'-CAGTGATGCTGTGCTATGAAT-3', reverse primers 5'-CAGATGCCTAAGTCTTCCAC-3'; Twist forward primers 5'-AGGTA TCGGGGATGTC-3'; MMP-9 forward primers 5'-ACAGCC GCAGACTTTCTTT-3'; MMP-2 forward primers 5'-CGCC TTAACTGGAGCACA-3', reverse primers 5'-AGGTAT CCGGGGATGCC-3'; MMP-9 forward primers 5'-ACGG CAGACATCGTATCC-3', reverse primers 5'-CCAGG GACCACAAGTCG-3'; GAPDH forward primers 5'-CCT TCCGTGTCCCCA-3', forward primers 5'-GCGCTGTC TACCAACCTTC-3'. GAPDH served as the internal control. All experiments were repeated 3 times.

Xenograft Tumor Growth Assay
Female BALB/c nude mice (4–6 weeks; n = 6/group) were purchased from Shanghai Laboratory Animal Center (Shanghai, China) and raised in specific pathogen-free conditions. SKOV3 cell lines were incubated for 24 h after gas treatment with or without 5.1% sevoﬂurane stimulation. Then, the SKOV3 cell lines were prepared as a 2 × 10⁶ cells/mL single-cell suspension. The female BALB/c nude mice were hypodermic injected with the cell suspension (0.2 mL/mouse) and divided into 2 groups: Control group and 5.1% sevoﬂurane group. The tumor volume was measured every 3 days, and the tumor volume (V) was calculated according to the formula: V = 1/2ab² (mm³). After 3 weeks, the mice were killed by air embolization and the tumor weight was calculated. The use and care of animals was carried out under the guidelines approved by the Institutional Animal Care and Use Committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University.

Statistical Analysis
SPSS 19.0 was processed for statistical analysis. The measurement data were expressed as mean ± standard deviation (SD). Student’s t-test and one-way ANOVA were, respectively, used to compare the differences of two independent samples and multiple groups. \( P<0.05 \) was considered statistically significant.

Result
Sevoﬂurane Inhibited Proliferation and Promoted Apoptosis in OC Cell Lines
At the beginning of this study, the OC cell lines SKOV3 and OVCAR3 were both treated without or with different concentrations of sevoﬂurane (1.7%, 3.4% or 5.1%). Then, the cell proliferation activity of SKOV3 and OVCAR3 was determined using MTT assay. The data from MTT assay exhibited that sevoﬂurane treatment signiﬁcantly inhibited the cell proliferation in SKOV3 and OVCAR3 cell lines compared to the control group (\( P<0.01, \) Figure 1A). And the inhibitory effect of sevoﬂurane on OC cells proliferation was dose-dependent (Figure 1A). We also checked the effect of sevoﬂurane treatment on OC cell apoptosis and found that sevoﬂurane notably enhanced the SKOV3 and OVCAR3 cells apoptosis compared with the control group on a dose-dependent (\( P<0.01, \) Figure 1B). Above data investigated that sevoﬂurane treatment could inhibit proliferation and promote apoptosis in OC cells.

Sevoﬂurane Inhibited Migration and Invasion in OC Cell Lines
To further explore the influence of sevoﬂurane on OC cell lines activities, transwell assay was employed. In SKOV3 cell lines, the results from transwell assay showed that cell migration and invasion activities were both dramatically decreased in sevoﬂurane groups compared to that in the control group (\( P<0.01, \) Figure 2). Likely, sevoﬂurane also obviously reduced OVCAR3 cells migration and invasion compared with the control group (\( P<0.01, \) Figure 2). All of these effects of sevoﬂurane on SKOV3 and OVCAR3 cells were in positive correlation with sevoﬂurane concentration. These findings confirmed that sevoﬂurane suppressed OC cell migration and invasion viability.

Effect of Sevoﬂurane on Proteins Expressions of Human OC Cell Proliferation and Invasion
To deﬁne the effects of sevoﬂurane on OC cells proliferation, apoptosis and invasion, the mRNA expression levels of proliferation-related factor (PCNA), apoptosis-related factor (caspase-3) and invasion-related factors (Twist, MMP-2 and MMP-9) were determined using qRT-PCR. As shown as Figure 3A, PCNA, Twist, MMP-2 and MMP-9 mRNA expressions were signiﬁcantly decreased while caspase-3 expression was markedly increased in sevoﬂurane groups compared to that in the control group (\( P<0.01, \)) Moreover, the protein expressions of PCNA, Twist, cleaved-caspase-3/caspase-3, MMP-2 and MMP-9 were assessed by Western blot. Similar to the results of qRT-PCR, PCNA, Twist, MMP-2 and MMP-9 expressions were inhibited as well as cleaved-caspase-3/caspase-3 expression was accelerated after sevoﬂurane treatment (Figure 3B). And the effects of sevoﬂurane on these
factors' expressions were positively related to the dose of sevoflurane. These results suggested that sevoflurane-repressed cell proliferation, migration and invasion and promoted apoptosis in OC.

Sevoflurane Inhibited Mitogen-Activated Protein Kinase (MAPK) Signaling Pathway

To analyze the potential mechanism of sevoflurane effecting on OC cells, the expression levels of MAPK signaling pathway-related factors, including p-p38, p38, p-JNK, JNK, p-ERK and ERK, were detected via Western blot. Figure 4 displays that the expression of p-p38/p38 and p-JNK/JNK was both remarkably suppressed on a dose-dependent manner in SKOV3 and OVCAR3 cell lines of sevoflurane groups compared to that of the control group ($P<0.01$). Although sevoflurane significantly inhibited the expression of ERK in the 5.1% group, the effect of sevoflurane on the ERK expression was obviously lower than that on the p38 or JNK expressions in the 1.7% and 3.4% groups. These data elucidated that sevoflurane interdicted the phosphorylation of p38 and JNK of MAPK signaling pathway.

Sevoflurane Regulated the Development of OC by Targeting MAPK Signaling Pathway

To identify the functional mechanism of sevoflurane in OC, MAPK signaling pathway was inhibited by p38 inhibitor (SB203580) or JNK inhibitor (SP600125). SKOV3 cell lines (5.1% group) were used to proceed with this experiment and the cell activities were examined. The data from MTT assay showed that the proliferation activities in 5.1% + SB203580 and 5.1% + SP600125 groups of OC cells were significantly higher than that in 5.1% group ($P<0.01$, Figure 5A). After sevoflurane treating, the cell apoptosis of the 4 groups was detected using flow cytometry and we investigated that SB203580 or SP600125 treatments obviously recovered the cell apoptosis induced by sevoflurane in OC cell lines SKOV3 ($P<0.01$, Figure 5B). Furthermore, the migration and invasion of SKOV3 cells were evaluated through transwell assay. Figure 5C exhibits that both SB203580 and SP600125 treatments could partly abolish the decrease of migration and invasion which are induced by sevoflurane in OC cell lines. These results illustrated that sevoflurane affected the...
proliferation, apoptosis, migration and invasion of OC cell lines via regulating the MAPK signaling pathway.

Sevoflurane Inhibited OC Tumor Growth in vivo

To explore the effect of sevoflurane on OC in vivo, xenograft tumor growth assay was performed. OC cell lines SKOV3 with or without 5.1% sevoflurane treatment were injected into nude mice. As shown in Figure 6A, the tumor volume of 5.1% group was significantly smaller than that of the control group ($P<0.01$). Furthermore, the tumor size and weight of mice in 5.1% group were both less than that in control group ($P<0.01$, Figure 6B and C). These data suggested that sevoflurane treatment could inhibit OC tumor growth in vivo.

Discussion

OC is one of the most common malignant tumors in female reproductive system. In recent years, the incidence of OC has been increasing year by year. The location of OC is hidden, which is the main cause of the death of gynecological malignant tumors. At present, the early diagnosis of OC is still a worldwide problem. Most patients with OC cannot be diagnosed until the advanced stage of the disease. Mortality and recurrence rate of OC patients are high. Platinum chemotherapeutic drugs are the first choice for OC treatment, it binds with DNA to form intra-and/or inter-chain adducts, which prevents the normal synthesis and replication of DNA, cause damage to genetic materials, induce cancer cell death and play an anti-tumor role. At present, the basic treatment principle of OC is extensive cytoreductive surgery combined with cisplatin and paclitaxel chemotherapy. However, OC is prone to chemotherapy resistance and recurrence of cancer focus. The recurrence rate of this scheme is as high as 70% in 2 years after the operation. In recent years, the exploration of effective new drugs for the treatment of OC has become the focus of OC research. Fu et al reported that metformin, an antidiabetic drug, could alleviate OC cell proliferation via suppressing m-TOR/PI3K/Akt pathway and inducing G2/M cell cycle interdict. Xu et al confirmed that Dihydrorymyricetin, a natural product which was isolated from Ampelopsis grossedentata, could inverse multi-drug resistance against OC cells and promote cell apoptosis by inhibiting survivin expression. However, the clinical diagnosis and treatment of OC are still difficult. Therefore, there is an urgent need to find effective new drugs for the treatment of OC, which is of great significance to improve the quality of life and survival rate of patients with OC.

Sevoflurane was usually well known as a common anesthetic in clinical practice. In recent years, scholars...
have gradually understood the therapeutic effects of sevoflurane in various tumors. Liu et al. found that sevoflurane inhibited breast cancer cell proliferation by triggering cell G1 phase arrest and promoting the expression level of miR-203. Yang et al. treated head and neck squamous cell carcinoma (HNSCC) cell lines FaDu and CAL-27 with sevoflurane and proved that sevoflurane-repressed cell viability by modulating HIF-1α signaling pathway in HNSCC cells. Liang et al. reported that an induction of cell apoptosis and a suppression of cell proliferation were observed in lung cancer cells which were treated with sevoflurane, and cell cycle arrest was also occurred. In the previous study, volatile anaesthetics were reported to be related to the expression of metastatic proteins and genes in OC, and inhalational anaesthetics might have a profound and distinct effect on OC tumor growth. However, the role and mechanism of sevoflurane in OC are still unclear. In the current study, we treated SKOV3 and OVCAR3 cell lines with different concentrations of sevoflurane, and found that sevoflurane significantly inhibited cell proliferation, migration and invasion and induced apoptosis of the OC cell lines. And, the effects of sevoflurane on OC cells were all positively correlated with the dose of sevoflurane. Moreover, we also proved that sevoflurane treatment suppressed OC tumor growth in vivo.

PCNA and caspase-3 were, respectively, reported to be related to cell proliferation and apoptosis, as well as Twist, MMP-2, MMP-9 and Twist, MMP-2, MMP-9 were identified as cell invasion-related factors. In this study, the expression levels of PCNA, caspase-3, Twist, MMP-2 and MMP-9 were detected by qRT-PCR and Western blot. Data from qRT-PCR and Western blot showed that sevoflurane could suppress the PCNA, Twist, MMP-2 and MMP-9 expression and enhanced the cleaved-caspase-3/caspase-3
It also indicated that sevoflurane-repressed proliferation and invasion and induced apoptosis in OC cell lines.

MAPK is an important pathway of intracellular signal transduction, an intersection point and common pathway of multiple signal transduction, which is widely distributed in the cytoplasm and transmits extracellular stimulus signals to the nucleus, and it can regulate of cell proliferation, differentiation and stress.33 P38 and JNK were reported to have belonged to principal MAPK pathways,34,35 and the expressions of p38 and JNK were assessed in our study. Dramatically decreases of p-p38/p38 and p-JNK/JNK expressions were observed in OC cells of sevoflurane groups compared to that of the control group.

Figure 4 Sevoflurane inhibited MAPK pathway-related proteins expressions. The protein expressions of MAPK pathway-related proteins (p-p38/p38, p-JNK/JNK and p-ERK/ERK) were detected using Western blot. Data were shown as mean ± SD. *P<0.05, **P<0.01 vs Control group.

Figure 5 Sevoflurane targeted MAPK signaling pathway to regulate OC cells development. SKOV3 (with 5.1% sevoflurane) cell lines were treated with p38 inhibitor (SB203580) or JNK inhibitor (SP600125) and divided into 4 groups included Control group, 5.1% (sevoflurane) group, 5.1% + SB203580 group and 5.1% + SP600125 group. (A) The cell proliferation activity of SKOV3 cell lines was detected using MTT assay. (B) The cell apoptosis of SKOV3 cell lines was determined using flow cytometry. (C) The cell migration and invasion of SKOV3 cell lines were measured using transwell assay. Data were shown as mean ± SD. *P<0.05, **P<0.01 vs Control group.
To verify whether sevoflurane affected OC cell lines through modulating MAPK pathway, p38 inhibitor SB203580 and JNK inhibitor SP600125 were used to treat SKOV3 cells with 5.1% sevoflurane. After that, SB203580 and SP600125 treatments both markedly accelerated proliferation, migration and invasion and blocked apoptosis in SKOV3 cells compared to the sevoflurane group. These results suggested that the therapeutic effect of sevoflurane on OC was significantly reduced when the MAPK pathway was blocked. Therefore, sevoflurane-regulated cell phenotype of OC cells via p38 and JNK MAPK signaling pathway.

In conclusion, we demonstrated that sevoflurane-inhibited proliferation, migration and invasion as well as facilitated apoptosis on a dose-dependent manner in OC cell lines via regulating p38 and JNK MAPK signaling pathway. It provided a novel strategy for the treatment of OC and a theoretical basis for the application of sevoflurane in OC therapy. However, the research of the potential influence of sevoflurane on OC biology is just beginning. Before making any decision to change the current clinical practice, a large number of preclinical and clinical studies are needed.

Availability of data and materials
All data generated and/or analyzed during this study are included in this published article.

Author Contributions
Both authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

Disclosure
The authors declare that they have no competing interests.

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