Effect of arsenic trioxide on cervical cancer and its mechanisms

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Abstract. Cervical cancer is one of the most common types of gynecological tumor, and thus identifying complementary or substitute treatment methods to treat cervical cancer is important. The present study aimed to evaluate the effect of arsenic trioxide (ATO), a traditional Chinese medicine, on cervical cancer cells and its underlying mechanism. MTT, colony formation and Transwell assays were performed to investigate the effects of different concentrations of ATO on cell proliferation and invasion, respectively. Western blotting and reverse transcription-quantitative PCR were applied to measure hypoxia-inducible factor-1α expression (HIF-1α) expression following ATO treatment. Finally, the effects of HIF-1α knockdown on cervical cancer cell proliferation, apoptosis and invasion were evaluated. The results demonstrated that ATO could inhibit cell proliferation and invasion. Moreover, ATO could induce reactive oxygen species production in a time- and dose-dependent manner. ATO could also promote the apoptosis of cervical cancer cells via HIF-1α. Therefore, the present study may provide a theoretical basis for identifying effective molecular targets for the prevention and treatment of cervical cancer.

Introduction

Cervical cancer is the 4th most common cancer type worldwide and is a prevalent type of malignant tumor in the female reproductive system (1). There are ~500,000 new cases annually worldwide, where the incidence is rising annually with diagnosis being common among younger women (2,3). Surgery plus radiotherapy can significantly improve the treatment effect of early-stage cancer, but for the late stage, the treatment effect of patients with relapse and metastasis remains at the bottleneck stage, and the 1-year survival rate of patients with late-stage cancer is <20% (4,5). Therefore, it is important to identify the molecular pathogenesis of cervical cancer, in order to develop complementary or substitute treatment methods, as well as more effective strategies to prevent cervical cancer, which will be of great theoretical and practical significance to improve the quality of life of patients with this disease worldwide.

Currently, comprehensive treatments, including surgery, lack a breakthrough in the curative effect of advanced and recurrent cervical cancer, and thus researchers have investigated traditional Chinese medicine (6,7). Arsenic is a traditional Chinese medicine that has three inorganic forms: Red arsenic (As2S3), yellow arsenic (As2S5) and white arsenic [As2O3; arsenic trioxide (ATO)], which are produced by oxidation of arsenic at high temperatures (8). In the mid and late 20th century, Chinese scholars first identified that ATO had significant clinical efficacy for the treatment of acute promyelocytic leukemia, and it is now used in clinical practice (9,10). Recently, additional research has examined solid tumors outside the blood system, and observed a good treatment efficacy in response to ATO (11). Previous studies have reported that ATO exerts antitumor effects on various solid tumors, including liver, breast, osteosarcoma and ovarian cancer, and its possible mechanism of action has also been investigated (12-14). However, its efficacy against cervical cancer has yet to be elucidated, where the underlying mechanism of action remain unknown.
Reactive oxygen species (ROS) is a general term for several active substances formed by oxygen, and the metabolism of ROS is closely associated with numerous metabolic regulatory activities, such as glucose metabolism, in the human body (15,16). The vast majority of oxygen accepts four electrons to combine with $H^+$ to form water, while some oxygen molecules produce ROS in a single-valent reduction form (17). ROS are important signaling molecules that not only regulate the structure and function of blood vessels, but also serve an important role in regulating cell proliferation, migration and differentiation by effecting the cellular proteins, lipids and nucleic acids (18-20).

Previous studies have revealed that hypoxia-inducible factor-1α (HIF-1α) is an important factor in promoting cell proliferation, invasion and metastasis of malignant tumors (21,22). Moreover, ATO can suppress HIF-1α production to serve an antitumor function in lung carcinoma and breast cancer cells (23,24). However, to the best of our knowledge, the relationship of ATO and HIF-1α has been rarely studied in cervical cancer.

The aim of the present study was to identify a novel strategy for the prevention and treatment of cervical cancer. In the present study, the effect of different times and concentrations of ATO treatment on cervical cancer was investigated. SiHa cells were used to examine the IC$_{50}$ of ATO in cervical cancer cells, as well as to assess the effects of ATO on cell proliferation and invasion. Furthermore, ROS and HIF-1α production were detected to elucidate the mechanism of ATO.

Materials and methods

Materials. The SiHa cervical cancer cell line and 293T cells were purchased from Shanghai Bioengineering Co., Ltd. FBS and RPMI-1640 medium were purchased from Gibco (Thermo Fisher Scientific, Inc.), while the BeyoFast™ SYBR-Green quantitative PCR premix Mix kit, Hoechst 33258 Staining kit (cat. no. C0003), RIPA lysis buffer (cat. no. P0013B), bicinchoinic acid (BCA) protein quantitative kit and MTT kit were all purchased from Beyotime Institute of Biotechnology. PVDF 45-µm non-sterile 50/pk membranes were purchased from EMD Millipore. ATO (white) was purchased from Sigma-Aldrich (Merck KGaA) and the Annexin V-FITC/PI double-staining kit (cat. no. 40302ES20) was purchased from Shanghai Yeasen Biotechnology Co., Ltd.

Cell culture and treatment. ATO powder (0.1 g) was dissolved in 10 ml sodium hydroxide (5 mol/l) before the pH of the solution was adjusted to neutral with hydrogen chloride. ATO was wrapped in foil and kept at -4°C. Before it was used in cell culture, ATO solution was diluted to the appropriate concentrations (0.5, 10, 15 and 20 µM) with PBS and filtered with a 0.22-µm filter. SiHa cells were cultured in RPMI-1640 medium containing 10% FBS at 5% CO$_2$, 37°C and saturated humidity, and the medium was changed once daily. After the cells entered the logarithmic long-term phase of growth, they were digested using 0.25% pancreatin at 37°C for 3 min. The cells were inoculated into 96-well (5x10$^4$ cells/well), 24-well (2x10$^5$ cells/well) or 6-well (1x10$^6$ cells/well) plates and cultured for 24 h at 37°C, before being added with different concentrations of ATO (0.5, 10, 15 and 20 µM) at the indicated time points (0, 6, 12 and 24 h) and incubated at 37°C.

Proliferation inhibition assay. The cells were inoculated at a density of 1x10$^4$ cells/well in 96-well culture plates. Each group had three compound wells and continued to be cultured after adding different concentrations of ATO (0, 5, 10, 15 and 20 µM) for 0, 6, 12 and 24 h. After the culture solution was removed at different time-points, 10 µl MTT (5 mg/ml) was added to the culture. After culturing for 4 h, the culture was terminated. After the culture solution was absorbed, 100 µl DMSO was added to each well at 37°C for 10 min. The optical density (OD) values of each group were measured at 570 nm using an enzyme labeling instrument (Type no. Sunrise; Tecan Group, Ltd.). The cell survival curves were plotted with the culture time as abscissa and OD as the ordinate.

Detection of ROS content. Intracellular ROS, such as H$_2$O$_2$ and •OH, were determined on the basis of fluorescent light detection using an oxidation-sensitive fluorescent probe dye, 2',7'-dichlorodihydrofluorescein diacetate (H$_2$DCFDA; Invitrogen; Thermo Fisher Scientific, Inc.) (25). The experiment was performed according to the manufacturer's protocols (26). In brief, 1x10$^6$ cells were incubated with the indicated concentrations (0, 5, 10, 15 and 20 µM) of ATO for 0, 6, 12 and 24 h at 37°C. The cells were then washed in PBS and incubated with 20 µM H$_2$DCFDA at 37°C for 30 min according to the manufacturer's instruction. Fluorescence was detected at 488 nm excitation wavelength and 525 nm emission wavelength using an enzyme labeling instrument (Type no. Sunrise; Tecan Group, Ltd.).

Colony formation assay. Cells in the logarithmic growth phase were prepared into single-cell suspensions and counted. In total, 1,000 cells were inoculated in each 60-mm culture dish and cultured in a 5% CO$_2$ incubator for 37°C. The medium was changed every 3 days and then removed. Cells were washed three times with PBS, fixed with 100% anhydrous methanol for 15 min at room temperature and stained with 0.1% crystal violet solution for 15 min at room temperature. Subsequently, the number of colonies containing >15 cells was counted under a light microscope (magnification, x400; Carl Zeiss AG).

Cell invasion assay. The final mass concentration of Matrigel was adjusted to 1 mg/ml using 4°C precooled serum-free medium. The upper Transwell chamber with 8-µm pore-size filters (Corning, Inc.) was coated with Matrigel for 3 -5 h and RPMI-1640 medium was added to the upper layer of the chamber, and RPMI-1640 medium containing 10% FBS at 5% CO$_2$, 37°C and saturated humidity, and the medium was changed once daily. After the cells entered the logarithmic long-term phase of growth, they were digested using 0.25% pancreatin at 37°C for 3 min. The cells were inoculated into 96-well (5x10$^4$ cells/well), 24-well (2x10$^5$ cells/well) or 6-well (1x10$^6$ cells/well) plates and cultured for 24 h at 37°C, before being added with different concentrations of ATO (0.5, 10, 15 and 20 µM) at the indicated time points (0, 6, 12 and 24 h) and incubated at 37°C.
with water several times, dried while inverted and mounted with a coverslip and neutral gum. Under a light microscope (magnification x200; Carl Zeiss AG), six visual fields were randomly selected, and the number of cells stained with crystal violet solution (i.e., the number of invasive cells) was counted.

**Western blot analysis.** The RIPA lysis buffer (including 1 μM PMSF protease inhibitor) was added to SiHa cells at logarithmic growth phase and then placed on ice for 40 min. The supernatant was collected via 13,000 x g centrifugation for 20 min at 4°C. The total cell protein was obtained, and the protein concentration was determined using the BCA method. After separating the protein (40 μg protein/lane) on a nitrocellulose membrane with 10% SDS-PAGE, membranes were blocked in 5% skimmed milk powder at room temperature overnight on a rocker at 4°C, followed by washing three times with PBS-0.1% Tween-20 (PBST) for 10 min each. The polyclonal goat anti-mouse horseradish peroxidase-conjugated secondary antibody (1:5,000; cat. no. ab6789; Abcam) was added and the membranes were incubated with PBS-0.1%Tween-20 (PBST) for 10 min each time. Odyssey infrared laser scanning imaging (LI-COR Biosciences) and ImageJ 6.0 (National Institutes of Health) were used to analyze the results.

**Reverse transcription-quantitative PCR (RT-qPCR) analysis.** The total RNA of the SiHa cells from each group was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the RNA purity and concentration were determined using a UV spectrophotometer (Tecan Group, Ltd.). The RNA to cDNA EcoDry™ Premix (cat. no. 639549; Takara Biotechnology Co., Ltd.) was used to reverse-transcribe RNA using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and the RNA purity and concentration were determined using an ultraviolet spectrophotometer (Tecan Group, Ltd.). The three plasmids aforementioned (total DNA 1.5 μg), recombinant lentivirus plasmid (1.5 μg) and Lipofectamine® 2000 (6 μl; Invitrogen; Thermo Fisher Scientific, Inc.) were then mixed. After being kept at room temperature for 20 min, the mixture was added to the culture medium of 293T cells drop by drop for transfection. The culture medium was replaced with fresh medium after 6 h, which then continued to cultivate for 24 or 48 h. The supernatant was collected and the lentivirus particles were harvested by ultracentrifugation (2 h at 50,000 x g; 4°C). SiHa cells were infected [multiplicity of infection (MOI)=10]. After 12-16 h, the medium was replaced with fresh medium. The cells were harvested for subsequent experimentation after 48 h. The RNA single-strand template sequences for the two pairs of interference fragments shRNA1 and shRNA2 are as follows: HIF-1α shRNA sequence 1 forward, 5'-CCGGCTGCC CTTCAGCATAAATTTGCTCCATATGGTCTGGAGAACACA AAGATACGGGTGTCC-3' and reverse, 3'-AATTCAGAAA ACCGCAATTCAATTTGACACTCTCGGTTAATATA GTCATATTGGTCCAGG-5'; and HIF-1α shRNA sequence 2 forward, 5'-TGCAACATTTTATGATTAGACCAACAATCA GTGAGAGTCAGATAACGTTATTCGGTTTGG-3' and reverse, 3'-AATTCAAAAAACAGAAGTTCCTCTACCTAGTA AAATATGTTCGTGATCGATTAAAAATTTGG-5'.

**Hoechst staining and flow cytometry assay.** Cells in the logarithmic growth phase were collected, and a single-cell suspension with a concentration of 2,5x10² cells/ml was generated. Then, a 200 μl suspension was added to each well of a 96-well plate. After the cells adhered, lentivirus (MOI=10) was used to infect SiHa cells, whilst the control group, which was transfected with the negative control lentiviral vector, was set up, with three wells for each group. After incubating for 72 h at 37°C and 5% CO₂, the culture medium was aspirated and the cells were fixed in 4% precooled paraformaldehyde at 37°C for 30 min and stained at 37°C with Hoechst 33258 staining solution for 5 min (28). The cells were then observed and imaged using.

**Table I. Reverse transcription-quantitative PCR primer information.**

| Gene | Primer | Primer sequence | Annealing temperature, °C |
|------|--------|----------------|--------------------------|
| HIF-1α | F | 5'-ACAAGTCACCACAGGACAG-3' | 56.91 |
| | R | 5'-AGGGAGAAATCAAGTGC-3' | 51.46 |
| GAPDH | F | 5'-AGGGAGAAAATCAAGTG-3' | 62.14 |
| | R | 5'-GGGGTGCGTTGATGGCAACA-3' | 60.75 |

F, forward; R, reverse; HIF-1α, hypoxia-inducible factor-1α.

**Construction of HIF-1α knockdown lentivirus plasmid.** The recombinant lentivirus plasmid pLKO.1-HIF-1α short hairpin (sh)RNA sequence 1, pLKO.1-HIF-1α shRNA sequence 2 and negative control plasmid pLKO.01-scrambled-shRNA were designed and synthesized by Cyagen Biosciences Inc. 293T cells were cultured in RPMI-1640 medium containing 10% PBS at 5% CO₂, 37°C. To produce virus, 293T cells (1.6x10⁶ cells/well) were seeded into 6-well plates prior to transfection. The ratio of pMDL (0.75 µg), VSVG (0.45 µg), and pRSV-Rev plasmids (0.3 µg) were 5:3:2, which were all purchased from Shanghai GeneChem Co., Ltd. The three plasmids aforementioned (total DNA 1.5 μg), recombinant lentivirus plasmid (1.5 μg) and Lipofectamine® 2000 (6 μl; Invitrogen; Thermo Fisher Scientific, Inc.) were then mixed. After being kept at room temperature for 20 min, the mixture was added to the culture medium of 293T cells drop by drop for transfection. The culture medium was replaced with fresh medium after 6 h, which then continued to cultivate for 24 or 48 h. The supernatant was collected and the lentivirus particles were harvested by ultracentrifugation (2 h at 50,000 x g; 4°C). SiHa cells were infected [multiplicity of infection (MOI)=10]. After 12-16 h, the medium was replaced with fresh medium. The cells were harvested for subsequent experimentation after 48 h. The DNA single-strand template sequences for the two pairs of interference fragments shRNA1 and shRNA2 are as follows: HIF-1α shRNA sequence 1 forward, 5'-CCGGCTGCC CTTCAGCATAAATTTGCTCCATATGGTCTGGAGAACACA AAGATACGGGTGTCC-3' and reverse, 3'-AATTCAGAAA ACCGCAATTCAATTTGACACTCTCGGTTAATATA GTCATATTGGTCCAGG-5'; and HIF-1α shRNA sequence 2 forward, 5'-TGCAACATTTTATGATTAGACCAACAATCA GTGAGAGTCAGATAACGTTATTCGGTTTGG-3' and reverse, 3'-AATTCAAAAAACAGAAGTTCCTCTACCTAGTA AAATATGTTCGTGATCGATTAAAAATTTGG-5'.

**Hoechst staining and flow cytometry assay.** Cells in the logarithmic growth phase were collected, and a single-cell suspension with a concentration of 2.5x10² cells/ml was generated. Then, a 200 μl suspension was added to each well of a 96-well plate. After the cells adhered, lentivirus (MOI=10) was used to infect SiHa cells, whilst the control group, which was transfected with the negative control lentiviral vector, was set up, with three wells for each group. After incubating for 72 h at 37°C and 5% CO₂, the culture medium was aspirated and the cells were fixed in 4% precooled paraformaldehyde at 37°C for 30 min and stained at 37°C with Hoechst 33258 staining solution for 5 min (28). The cells were then observed and imaged using.
fluorescence microscopy (magnification, x200). For the flow cytometry detection, SiHa cells infected with lentivirus were harvested, washed with PBS and stained with a Annexin V-FITC and PI double-staining kit for 15 min for 37°C (29). Subsequently, these cells were analyzed via flow cytometry (BD FACSCalibur™; BD Biosciences) within 1 h. The results were evaluated by CellQuest Pro software (version 5.0; BD Biosciences). The apoptotic rate was calculated by the percentage of early and late apoptotic cells.

Statistical analysis. All experiments were repeated three times. Data are presented as the mean ± SD. Unpaired t-test was used to compare two groups, and one-way ANOVA followed by Bonferroni post hoc test was used to compare multiple groups. P<0.05 was considered to indicate a statistically significant difference. All data were analyzed using the SPSS 20 (IBM Corp.) software.

Results

Cell viability inhibition rate and ROS content in cells. The 50% inhibitory concentrations (IC_{50}) is the dose of ATO against proliferation of cervical cancer cells within the prescribed time (30). The cell viability inhibition rate was measured using the MTT method after the cells were treated at different ATO concentrations (0, 5, 10, 15 and 20 µM), which demonstrated the inhibition rate to be dependent on the concentration, with the highest rat observed in the 20 µM group. Besides, it was found that with the prolongation of time, the inhibition rate of the ATO first increased, then decreased (Fig. 1A). Subsequently, the inhibition rate remained at a relatively stable level. At 6 h, the inhibition rate was highest in all groups. Thus, ATO had a time- and dose-dependent effect on cell viability.

After the cells were treated with different ATO concentrations for 0, 6, 12 and 24 h, the intracellular ROS content was detected. The ROS content first increased and then decreased in a time- and dose-dependent manner, and it was the highest at 6 h, which suggested that ATO inhibited the proliferation of cervical cancer cells by mainly relying on ROS production (Fig. 1B).

Colony formation assay and cell invasion. SiHa cells were treated with different concentrations ATO for 6 h, the colony formation and Transwell assays were performed to detect cell proliferation and invasion (Fig. 2A and B). When there was no drug treatment, the colony-forming ability of the cells was increased, whilst higher drug concentrations led
to markedly fewer numbers of cell colonies and lowered the invasive capabilities of cells. Therefore, the results suggested that ATO inhibited SiHa cell proliferation and invasion in a dose-dependent manner.

**HIF-1α expression with ATO treatment.** The relative expression of HIF-1α mRNA was measured with different concentrations of ATO. The expression of HIF-1α mRNA was significantly lower in the drug-treated groups (10, 15 and 20 μM) compared with the control group (P<0.05), but there was no significant difference in the expression of HIF-1α mRNA among these three experimental groups (Fig. 3A). Thus, ATO significantly inhibited the expression of HIF-1α mRNA at 6 h. The expression of HIF-1α protein was also significantly lower in the 10, 15 and 20 μM groups (Fig. 3B and C) compared with that in the control group, which was in line with the RT-qPCR results for HIF-1α mRNA expression.

**Colony formation and cell migration after knockdown of HIF-1α.** HIF-1α mRNA and protein expression levels were detected after infection with lentivirus. The expression levels of HIF-1α mRNA and protein were significantly lower in the HIF-1α shRNA 1 and shRNA 2 groups compared with the control group (Fig. 4). Moreover, HIF-1α shRNA 1 demonstrated a higher inhibitory effect compared with shRNA 2. Therefore, HIF-1α shRNA 1 was selected for subsequent experiments.

The results of the colony formation and cell migration experiments are presented in Fig. 5. Compared with the control group, the number of colonies formed in the HIF-1α knockdown group was markedly lower, indicating that the knockdown of the HIF-1α gene inhibited cell proliferation (Fig. 5A). Furthermore, the cell invasion rate was significantly decreased, suggesting that knocking down HIF-1α reduced the cell invasive ability (Fig. 5B and C).
Apoptosis assay after HIF-1α knockdown. Lentivirus-infected SiHa cells were stained with Hoechst dye after 72 h. SiHa cells exhibited typical morphological changes indicating apoptosis, such as nuclear condensation and nucleosome and nuclear fragmentation, following knockdown of the HIF-1α gene (Fig. 6A). In addition, the apoptotic rate of cells after knockdown was ~50%, demonstrating that HIF-1α knockdown promoted cell apoptosis (Fig. 6B). Flow cytometric analysis was performed to further evaluate apoptosis, and it was identified that after knocking down the HIF-1α gene, the early and late apoptotic rate of SiHa cells was increased by ~3-fold compared with that in the control group (Fig. 6C and D), which was consistent with the aforementioned Hoechst staining results.

Discussion

Cervical cancer is one of the most common gynecological malignancies, which tends to be diagnosed in younger women, and ~570,000 cases of cervical cancer and 311,000 deaths from the disease occurred in 2018 (31). The estimated age-standardized incidence of cervical cancer was 13·1 per 100,000 women globally (31). There is currently no established standard for...
effective treatment, and there is yet no commonly accepted highly effective drug. At present, the main treatment principles for cervical cancer are surgery, followed by radiotherapy and chemotherapy (32). Chemotherapy is of great significance in the treatment of cervical cancer, but the main application of chemotherapeutic drugs is not targeted therapy and there are a number of side effects (33). Chemotherapeutic resistance is a major challenge of recurrence and metastasis of cervical cancer (33). Therefore, identifying novel, high-efficiency and low-toxicity chemotherapy drugs will be the primary direction of future research (28,33).

ATO is extracted from the traditional Chinese medicine white arsenic, which is easy to obtain, cost-effective and convenient (34). Recent studies have reported that ATO not only serves an important role in the treatment of hematological malignancies, but also has a beneficial antitumor efficacy in solid tumors (11-13). However, its specific mechanism of action requires further investigation.

ATO-induced apoptosis is a complex process involving multiple targets, and is one of its main antitumor effects (35). Previous studies have revealed that the antitumor effect of ATO is closely associated with ROS (36,37). Moreover, the baseline levels of ROS can stimulate the mitosis, DNA synthesis and the proliferation of tumor cells (38). However, a high level of ROS can induce tumor cell apoptosis and cause tumor cell necrosis (39). It has been reported that an important early cell activity in ATO treatment of target cells is the change in the ROS level, and ATO can increase the level of ROS in cells by acting on multiple pathways, including the ATM-ATR-associated Chk pathway and caspase-3-dependent apoptosis (37,40). However, the exact mechanism of action of ATO in cervical cancer is yet to be fully elucidated (41).

In the present study, different concentrations of ATO were applied to SiHa cells to detect the inhibitory effect of ATO on cellular processes. The results demonstrated that the inhibition rate of 20 µM ATO was ~50% when the cells were treated for 6 h, and the inhibitory effect of ATO on cells was time- and dose-dependent. The level of ROS produced by ATO was also time- and dose-dependent, which indicated that ATO inhibited cervical cancer cellular functions via inducing ROS production in a time- and dose-dependent manner.

HIF-1α is an important factor in determining the prognosis of malignant tumors. For instance, excessive activation of this factor can promote the proliferation, infiltration and metastasis of tumor cells, as well as induce tumor cell resistance to chemotherapy and radiotherapy (42). However, to the best of our knowledge, the relationship of ATO and HIF-1α has been rarely studied in cervical cancer. In the present study, it was identified that ATO could inhibit the expression of HIF-1α, and that the knockdown of HIF-1α can effectively suppressed SiHa cell proliferation and invasion, and promote cell apoptosis.

In conclusion, with regards to the mechanism of action of ATO against cervical cancer, the present results suggested that ATO may inhibit cell proliferation and migration via inducing ROS and inhibiting HIF-1α production. The present study may provide a further theoretical basis for identifying effective molecular targets for the prevention and treatment of cervical cancer. However, the mechanism via which ATO induces ROS and suppresses HIF-1α, and its downregulation of cervical cancer are yet to be elucidated. In addition, additional animal experiments need to further verify the safety of ATO.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
LZ, YZ, YC and XY conceived and designed the research. LZ, YZ, JK, YW, SX, LZ and MY conducted the experiments. All authors analyzed and interpreted the data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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