Spermidine-induced growth inhibition and apoptosis via autophagic activation in cervical cancer

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Abstract. Cervical cancer is the most common malignancy of the female reproductive tract, and the poor response to prophylactic vaccines and the toxicity of high-dose chemotherapeutic drugs have limited their clinical application. Spermidine, a natural polyamine detected in all eukaryotic organisms, exhibits functions that promote longevity in multiple model systems and may constitute a promising agent for cancer treatment. However, the potential effectiveness of spermidine in cervical cancer has not yet been fully elucidated, and the underlying molecular mechanisms remain unclear. In the present study, we aimed to assess the effects of spermidine on proliferation and apoptosis of HeLa cells (a cervical cancer cell line). Firstly, CCK-8 and flow cytometric assays revealed that spermidine reduced the proliferation of HeLa cells in a dose-dependent fashion by arresting the cell cycle at the S phase. Secondly, flow cytometry incorporating Annexin V-FITC/PI-staining revealed that spermidine promoted the apoptosis of HeLa cells, and western blot analysis revealed that spermidine activated autophagy. Finally, spermidine-activated autophagy mediated the inhibition of cell proliferation by spermidine and spermidine-induced apoptosis in HeLa cells. Collectively, these results revealed a novel function for spermidine in inhibiting cellular proliferation and inducing apoptosis of HeLa cells by activating autophagy, which may have important implications for the use of spermidine in cervical cancer therapy.

Introduction

Cervical cancer is the most common malignancy of the female reproductive tract, and 95% of cases are caused by persistent infections with carcinogenic human papillomaviruses (HPV) (1). Cervical cancer accounts for 528,000 new cases and 266,000 deaths worldwide each year, more than any other gynecologic tumor (2). Effective preventive vaccines are available for the most important carcinogenic HPV strains, but their use remains poor (3). At present, the combination of radiotherapy and platinum-based chemotherapy is the gold-standard therapy for advanced cervical cancer (4). Although the addition of platinum-based chemotherapy to radiotherapy has increased the 5-year survival of advanced-stage cervical cancer patients (5), systemic toxicity limits the use of high-dose chemotherapeutic drugs. Therefore, new therapeutic strategies that are less toxic and that provide higher efficacy against cervical cancer are urgently needed. Natural components provide a great deal of opportunity for preventing or changing the pathogenic course of the disease in a safer and more cost-effective way.

Apoptosis and autophagy are both cellular degradative pathways essential for organismal homeostasis. Dysregulation of apoptosis leads to the accumulation of 'unwanted' cells and contributes to the development of cancer (6). Anticancer therapeutics may be used to promote apoptosis within cancer cells to cause the death of these cells. Current anticancer therapies, including many chemotherapeutic agents and ionizing radiation therapy, usually activate apoptosis and utilize the apoptotic machinery for killing cancer cells (7,8). Autophagy can remove damaged proteins and organelles, and it limits their cumulative deleterious effects intracellularly. Therefore, autophagic defects are found in many human tumors (9,10). In addition, excessive autophagy has been involved in autophagic cell death, which is characterized by morphological changes, such as the accumulation of autophagosomes in the cell (11). In contrast to the tumor-suppressor roles for autophagy, stress-activated autophagy may promote survival of tumor cells, especially when apoptosis is defective (6). Previous studies have suggested that fucoidan may exert its antiproliferative action in cultured HeLa cells by inducing apoptotic and autophagic cell death (12), and glaucocalyxin B may supress the proliferation of human cervical cancer cells *in vitro* through the induction of apoptosis and autophagy (13). Therefore, the study of the mechanisms underlying apoptosis and autophagy should provide therapeutic benefits in human cervical cancer.

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Spermidine, a natural polyamine detected in all eukaryotic organisms, exhibits many biological functions. It is involved in processes such as cellular proliferation, differentiation, tissue development, and carcinogenesis (14). Recent studies have revealed that macrophage ABHD5 suppressed spermidine production and subsequently promoted colorectal cancer growth (15). In addition, oral spermidine suppressed liver fibrosis and hepatocellular carcinoma in mice by activating MAP1S-mediated autophagy (16). It is suggested that spermidine constitutes a promising agent for the treatment of cancer. However, the potential effectiveness of spermidine in cervical cancer has not yet been fully elucidated, and the underlying molecular mechanism involved remains unknown.

The purpose of this study was to demonstrate the potential roles and molecular mechanisms underlying the effects of spermidine in cervical cancer. For this reason, we assessed its function in HeLa cells and found that spermidine strongly suppressed proliferation and induced apoptosis in HeLa cells. Additional mechanistic studies suggested that activation of autophagy mediated the inhibition of cellular proliferation by spermidine and spermidine-induced apoptosis in HeLa cells. Collectively, our results revealed the potent effects of spermidine in the treatment of cervical cancer and provided experimental evidence about the details of the underlying mechanisms.

Materials and methods

Reagents and cell culture. Spermidine was dissolved in dimethylsulfoxide (DMSO; both from Sigma-Aldrich; Merck KGaA, St. Louis, MO, USA). 3-Methyladenine (Sigma-Aldrich; Merck KGaA), an autophagy inhibitor, was used at a concentration of 4 mM in DMSO.

The human cervical cancer cell lines HeLa (HPV-18-infected cervical cancer cells), SiHa (HPV-16-infected cervical cancer cells), and C-33A (HPV-negative cervical cancer cells) were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Cells were grown in DMEM (GIBCO; Thermo Fisher Scientific, Inc., Waltham, MA, USA), supplemented with 10% fetal bovine serum (Beyotime Institute of Biotechnology, Haimen, China) and 100 U/ml streptomycin/penicillin at 37°C in a humidified atmosphere containing 5% CO_2 in compressed air. When 80-90% confluence was reached, HeLa cells were digested with 0.25% trypsin (Beyotime Institute of Biotechnology) for subsequent experiments.

Cell proliferation assay. Cell viability with spermidine treatment was evaluated using CCK-8 colorimetric assay (Beyotime Institute of Biotechnology) (17). Briefly, cells were seeded in 96-well plates at a density of $2x10^4$ cells/well, at 37°C with 5% CO_2. After culturing cells to 80% confluence, the cells were exposed to varying concentrations of spermidine for 24, 48 or 72 h. CCK-8 was then added to each well followed by incubation, and the absorbance was assessed at 450 nm using a microplate reader (BioTek Instruments, Inc., Winooski, VT, USA). Cell viability was calculated using the following formula: Cell viability (%) = (OD treatment - OD blank)/(OD control - OD blank).

Quantitative real-time PCR (qRT-PCR) assay. HeLa cells were cultured with spermidine at 60, 120 and 180 μM for 24 h, respectively. The control cells were treated with 0.1% DMSO. Following spermidine incubation, the mRNA from HeLa cells was extracted (MagnaPure LC RNA Isolation Kit) and underwent reverse transcription into cDNA according to the Transcription High Fidelity cDNA Synthesis kit (both from Roche Applied Science, Penzberg, Germany), following the corresponding manufacturer's instructions. The expression levels were evaluated using a two-step qRT-PCR kit with SYBR®-Green (Takara Biotechnology Co., Ltd., Dalian, China) with a final volume of 20 μl (10 μl SYBR®-Green qPCR Mixture, 10 μM forward and reverse primers) on a 7500 Real-time PCR System (ABI). The threshold cycle values (Ct values) of the genes and internal control genes for the different samples were evaluated. The primer pairs are listed in Table I.

Cell cycle assay. HeLa cells in each group were trypsinized and fixed with 70% ethanol at 4°C for 12 h. Subsequent to washing with phosphate-buffered saline (pH 7.4), the cell cycle analysis was carried out with a Cell Cycle Detection kit (cat. no. C1052; Beyotime Institute of Biotechnology), according to the manufacturer's protocol. The kit contained binding buffer, propidium iodide (PI) staining buffer (20X), and RNase A (50X). In brief, the cells were stained in 500 μl of binding buffer containing 25 μl of PI staining buffer with 10 μl of RNase A for 30 min at 37°C in the dark. Cell cycle kinetics were detected with flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Results are expressed as the percentage of cells at each phase of the cell cycle.

Cell apoptosis assay. Cellular apoptosis was detected using the Annexin V-FITC/PI Apoptosis Detection Kit (MultiSciences Biotech Co., Ltd. Hangzhou, China). Briefly, after treatment with spermidine, HeLa cells were collected and washed twice with PBS. Cells at $5x10^5$ cells/ml were re-suspended in 400 μl of binding buffer with 5 μl of Annexin V-FITC and 1 μl of PI in the dark. After incubation for 15 min, cell apoptosis was assessed by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Western blot analysis. HeLa cells were lysed with RIPA lysate to extract total protein, and concentration was assessed by a bicinchoninic acid (BCA) protein assay kit (both from Beyotime Institute of Biotechnology). The same amounts of proteins were separated in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes (EMD Millipore, Bedford, MA, USA). Then, 5% skim milk was used to block proteins for 1 h. Subsequently, the blots were detected with specific primary antibodies against GAPDH (cat. no. AF1186), BAX (cat. no. AB026), Bcl-2 (cat. no. AB112), cleaved-caspase-3 (cat. no. AC033), (all 1:1,000; Beyotime Institute of Biotechnology), LC3B (cat. no. 3868S), Atg5 (cat. no. 2630S), Beclin 1 (cat. no. 3738S) (all 1:1,000; Cell Signaling Technology, Danvers, MA, USA) at 4°C incubation overnight. Then, the primary antibodies were washed away, and incubated with corresponding secondary antibodies [cat. no. A0208 (HRP-labeled goat anti-rabbit IgG (H+L) and

Table I. Primer sequences of ODC1, SRM, SMS, SSAT, SMOX, GAPDH.

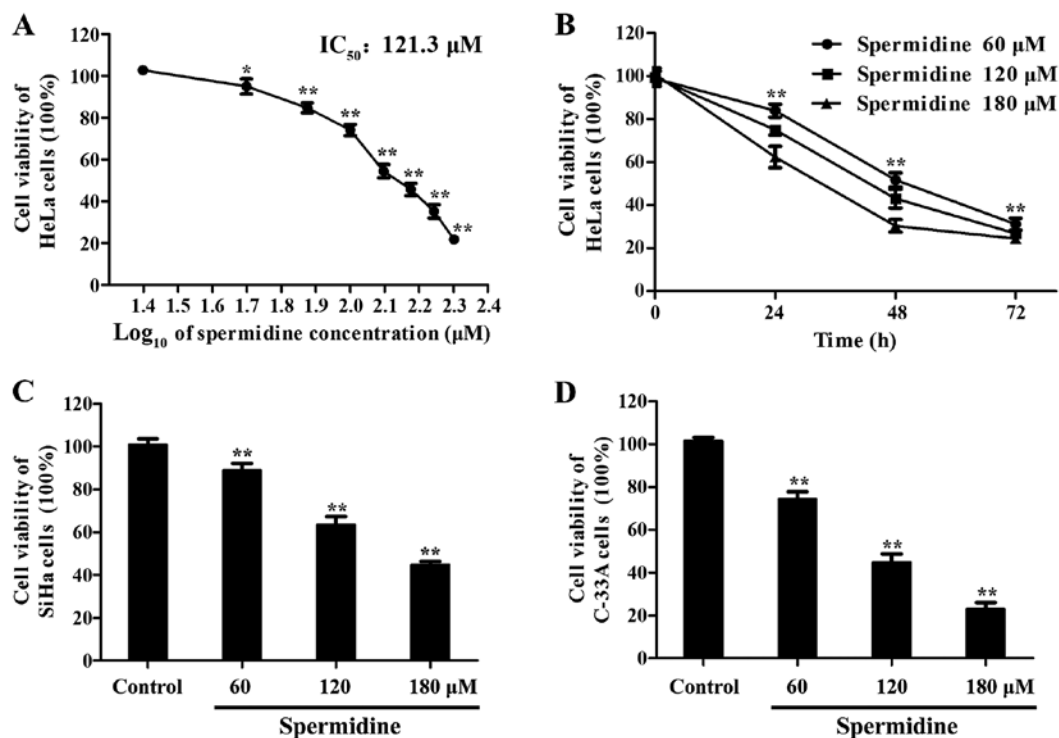| Gene	Forward	Reverse
ODC1	CAGCTTTCACGCTTGCAGTT	ATCTTCGTCATCAGAGCCCG
SRM	CCCTCCGTGGAGTCCGTGGTC	CTGGCAGGAACTTCTTGGAGACTTG
SMS	TGGAAATATTCTCATCCTTAGTGGG	CGGGTATATGCCAAATCACTCTCT
SSAT	GGCATAGGATCAGAAATTCTGAAGA	CTGCTACCAAGAAGTGCATGCTG
SMOX	CAATGCTGAAAGTCAAAATAGCGTG	CTCTGGGTCGTCAGGGTC ATTCC
GAPDH	CATCAATGGAAATCCCATCA	GACTCCACGACGTACTCAGC

Figure 1. Spermidine inhibits the proliferation of HeLa, SiHa, C-33A cell lines. (A) HeLa cells were cultured with an ascending concentration range of spermidine (from 0 to 200 μM) for 24 h and cell viability was detected by CCK-8 assay (n=6). Effects on cell viability were expressed as a function of μM drug concentration (log scale). Corresponding IC_{50} values were calculated with the appropriate software (GraphPad Prism). (B) HeLa cells were cultured with spermidine at 60, 120 or 180 μM, for 24, 48 and 72 h, respectively (n=6). (C) Spermidine treatment at 60, 120 and 180 μM in SiHa cells after 24 h was performed and cell viability was assessed by CCK-8 assays, respectively (n=6). (D) Spermidine treatment at 60, 120, 180 μM in C-33A cells after 24 h was performed and cell viability was assessed by CCK-8 assays, respectively (n=6). Data are the means ± SD. *P<0.05, **P<0.01, compared with the control. The controls were treated with 0.1% DMSO.

cat. no. A0216 (HRP-labeled goat anti-mouse IgG (H+L)] (1:5,000; Beyotime Institute of Biotechnology) for 1 h at room temperature. The enhanced chemiluminescence (ECL) solution (Qihai Biotec, Shanghai, China) was used to detect the target bands, and Gel-Pro-Analyzer software (Media Cybernetics, Inc., Rockville, MD, USA) was employed to analyze the gray values of the band. GAPDH served as a loading control.

Statistical analysis. Statistical analysis was performed using GraphPad Prism v. 5.0 software (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the means ± standard deviation (SD). All data presented represent results from at least 3 independent experiments. One-way analysis of variance (ANOVA) followed by the Tukey's multiple comparison test

was used to compare differences between groups. Statistical significance was defined as P<0.05.

Results

Spermidine reduces the proliferation of human cervical cancer cell lines. To study the effects of spermidine on the growth of human cervical cancer cell lines, we first performed CCK-8 analysis. The cells were treated with spermidine at different concentrations (0 to 200 μM) for 24 h, and then cell viability was assessed using CCK-8 assays. As shown in Fig. 1A, spermidine significantly inhibited the viability of HeLa cells in a dose-dependent manner. The IC_{50} values of spermidine for HeLa cells was 121.3 μM (Fig. 1A). Spermidine
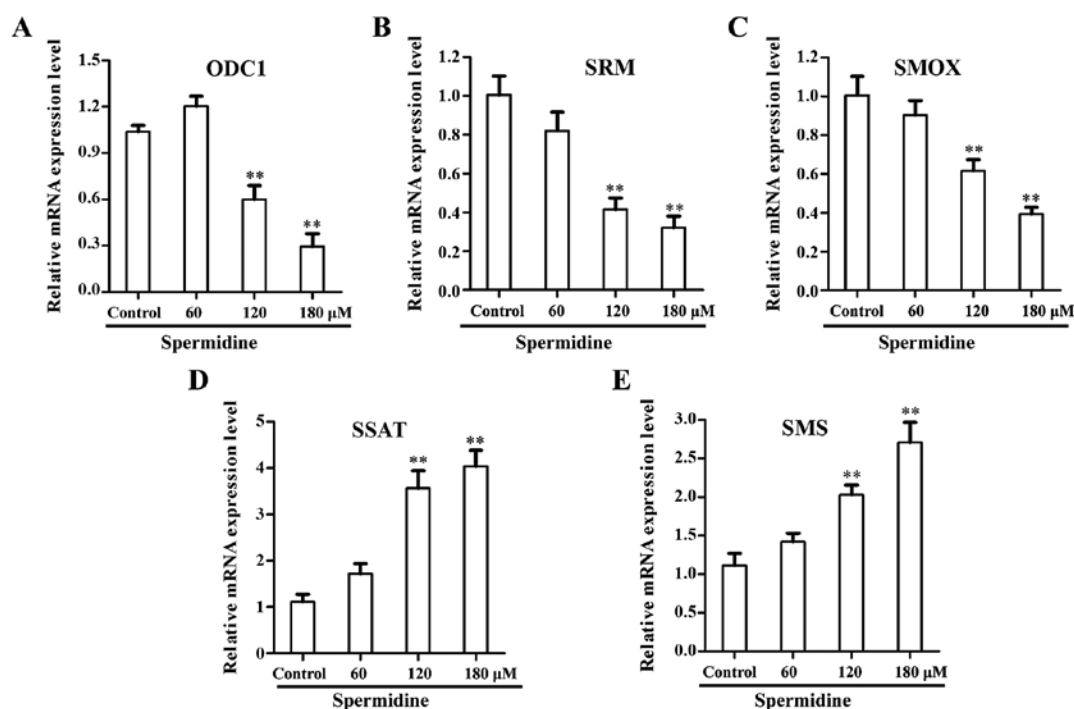
Figure 2. Spermidine affects the principal genes in the polyamine biosynthesis pathway of HeLa cells. (A-E) qRT-PCR detected ODC1, SRM, SMOX, SSAT and SMS mRNA expression levels and the relative expression was assessed by quantitative analysis. Data are expressed as the means ± standard deviation. **$P<0.01$, compared with the control. The controls were treated with 0.1% DMSO. ODC1, ornithine decarboxylase; SRM, spermidine synthase; SMOX, spermine oxidase; SSAT, spermidine/spermine N1-acetyltransferase; SMS, spermine synthase.

also significantly reduced the growth of HeLa cells in a time-dependent manner (for 24, 48 and 72 h) (Fig. 1B). To further confirm the inhibition of spermidine specifically in human cervical cancer cells, other human cervical cancer cell lines, including SiHa (HPV-16-infected cervical cancer cells) and C-33A (HPV-negative cervical cancer cells) were tested. Notably, spermidine inhibited the growth of both SiHa (Fig. 1C) and C-33A cell lines in a dose-dependent manner (Fig. 1D). We found that HeLa, SiHa and C-33A cell lines were all sensitive to spermidine-induced cytotoxicity. Collectively, these data revealed that spermidine reduced the viability of human cervical cancer cell lines.

Spermidine affects the mRNA levels of the polyamine biosynthesis pathway of HeLa cells. It has been well established that an increase in intracellular polyamine concentration is correlated with increased cell proliferation as well as tumorigenesis (18,19). Depletion of the intracellular polyamine pools using either a polyamine synthesis inhibitor or a polyamine analog invariably inhibits cell growth in various cancers (20-22). To confirm whether exogenous spermidine inhibited the accumulation of endogenous polyamines, we performed qRT-PCR to detect the messenger RNA (mRNA) expression levels of principal genes in the polyamine biosynthesis pathway. The results revealed that the mRNA level of ornithine decarboxylase (ODC1), spermidine synthase (SRM) and spermine oxidase (SMOX) were significantly decreased in HeLa cells after spermidine treatment (Fig. 2A-C), while the mRNA levels of spermidine/spermine N1-acetyltransferase (SSAT) and spermine synthase (SMS) were significantly increased in HeLa cells after spermidine treatment (Fig. 2D and E).

Collectively, these results revealed that exogenous spermidine inhibited the accumulation of endogenous polyamines.

Spermidine inhibits the viability of HeLa cells by arresting the cell cycle at the S phase. To further determine whether the viability of HeLa cells treated with spermidine was decreased, which was due to the reduction of cellular proliferation, we next investigated the effects of spermidine on cell cycle distribution. As shown in Fig. 3A-D, when cells were treated with 120 or 180 μM spermidine, the percentage of HeLa cells in the S phase was significantly increased from 23.78±0.75 to 37.42±1.49 and 38.65±1.05%, compared with the control group, respectively. In addition, the proportion of cells in the G2 phase was not altered (Fig. 3D). These results revealed that spermidine inhibited the proliferation of HeLa cells by arresting the cell cycle at the S phase.

Spermidine induces apoptosis of HeLa cells through the mitochondrial apoptotic pathway. To confirm whether spermidine inhibition of HeLa cell proliferation was caused by apoptosis, we used the Annexin V/PI Cell Apoptosis kit to detect the apoptotic effect of spermidine on HeLa cells by flow cytometric assays. We found that 120 and 180 μM of spermidine treatment significantly increased the apoptosis of HeLa cells compared with the control cells (Fig. 4A-D). To further investigate the promotion of apoptosis of HeLa cells by spermidine, western blot analysis revealed that the expression of Bcl-2 (an anti-apoptotic protein) in HeLa cells was significantly decreased after spermidine treatment (Fig. 5B and E); conversely, the expression of pro-apoptotic proteins cleaved-caspase-3 and BAX protein were significantly
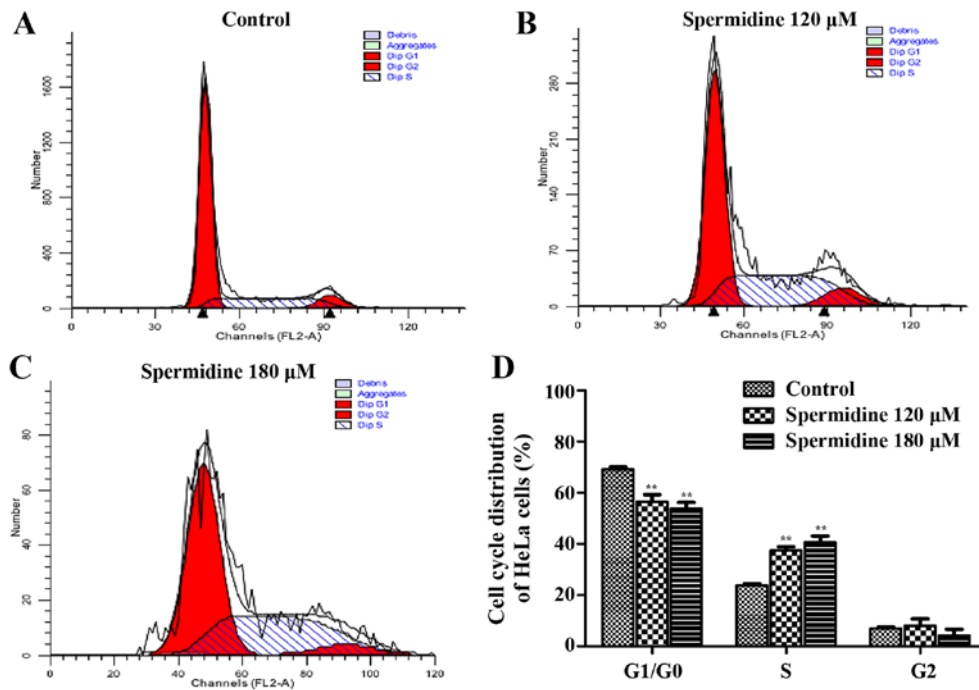
Figure 3. Spermidine reduces the viability of HeLa cells by arresting the cell cycle at the S phase. (A-C) Cell cycle distribution of HeLa cells was analyzed by flow cytometry, in cells treated with (A) the control, (B) 120 μM spermidine, or (C) 180 μM spermidine. (D) The percentage of cell cycle distribution of HeLa cells under various conditions (n=3) was represented by a column diagram. Data are expressed as the means ± SD. **P<0.01, compared with control. The controls were treated with 0.1% DMSO.
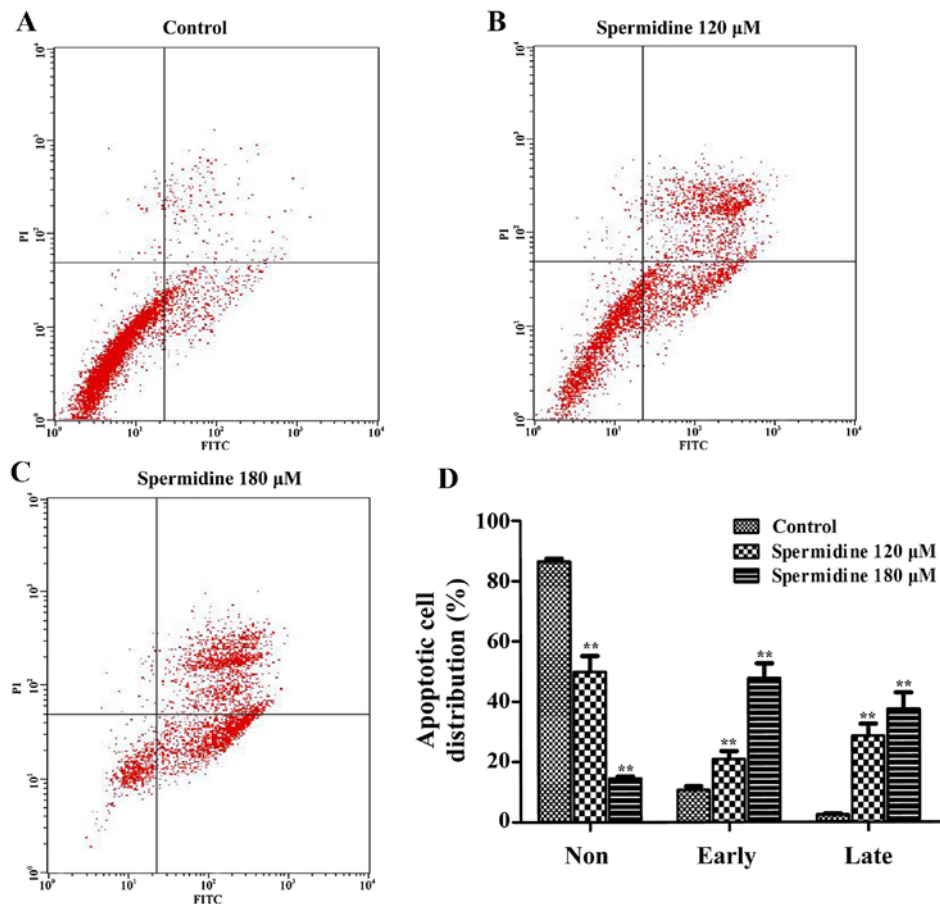

Figure 4. Spermidine induces apoptosis in HeLa cells. (A-C) Flow cytometric analysis of Annexin V-FITC/PI-stained HeLa cells treated with (A) the control (0.1% DMSO, (B) 120 μM spermidine, or (C) 180 μM spermidine. Cells were characterized as healthy cells (bottom left quadrant), necrotic (top left quadrant), early apoptotic (bottom right quadrant), or late apoptotic (top right quadrant). (D) Quantitative analysis of the apoptotic rates for HeLa cells under various concentrations of spermidine as displayed in A-C (n=3). Data are expressed as the means ± SD. **P<0.01, compared with the controls. The controls were treated with 0.1% DMSO.
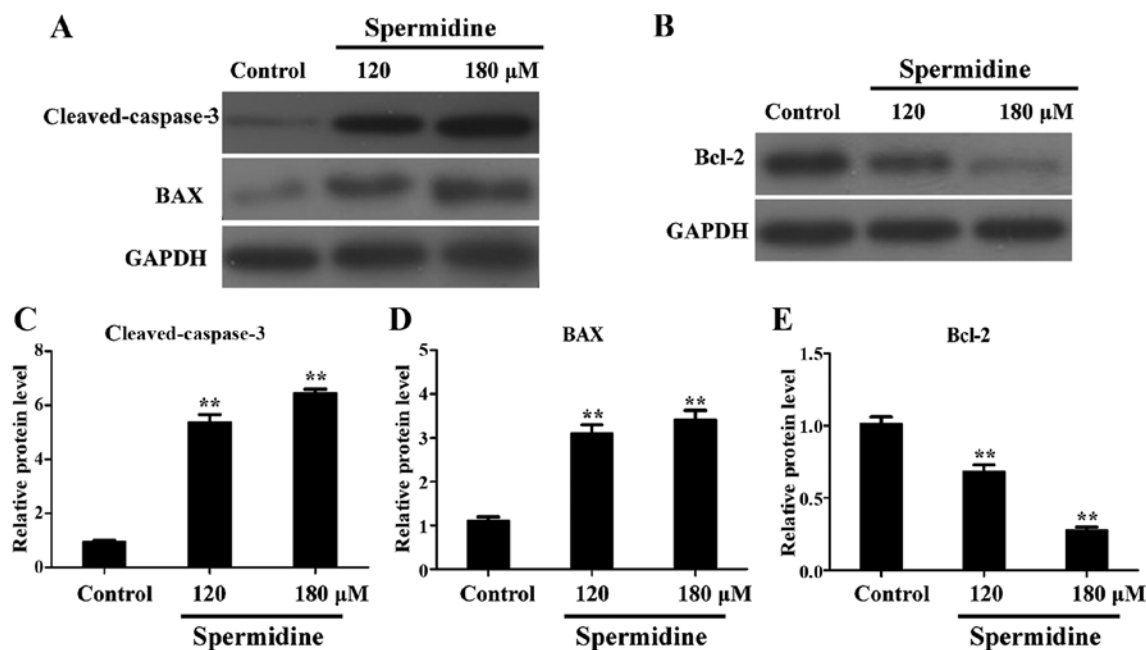
Figure 5. Spermidine affects the expression of c-caspase-3, BAX, and Bcl-2 in HeLa cells. (A-B) Western blot analysis detected the proteins of apoptosis, c-caspase-3, BAX, and Bcl-2 in cells treated with spermidine. (C-E) Quantitative analysis of the relative expression of (C) c-caspase-3, (D) BAX and (E) Bcl-2 as displayed in A and B (n=3). Data are expressed as the means ± SD. **$P<0.01$, compared with the controls. The controls were treated with 0.1% DMSO.
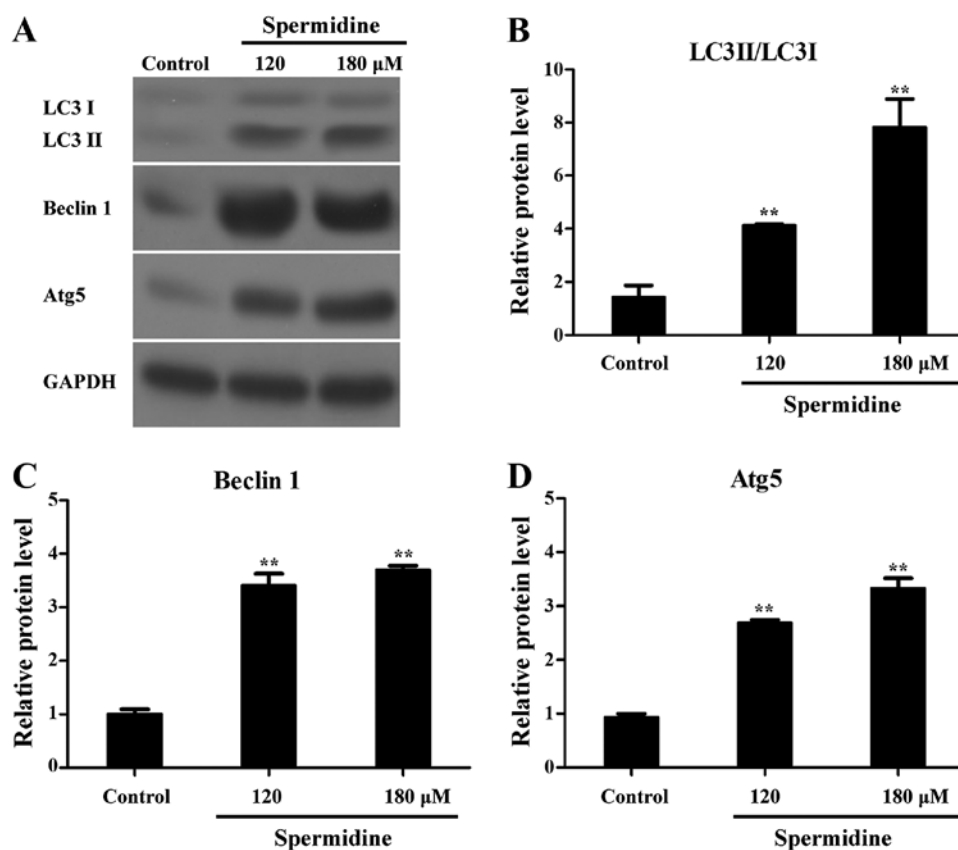

Figure 6. Spermidine affects the expression of LC3 II/LC3 I, Beclin 1, and Atg5 in HeLa cells. (A) Western blotting detected the proteins of autophagy, LC3 II/LC3 I, Beclin 1, and Atg5, in cells treated with spermidine. (B-D) Quantitative analysis of the relative expression of (B) LC3 II/LC3 I, (C) Beclin 1 and (D) Atg5 as displayed in A. (n=3). Data are expressed as the means ± SD. **$P<0.01$, compared with the controls. The controls were treated with 0.1% DMSO.

increased after spermidine treatment (Fig. 5A, C and D), compared with the controls. Collectively, these results strongly demonstrated that spermidine induced apoptosis of HeLa cells by triggering the mitochondrial apoptotic pathway.
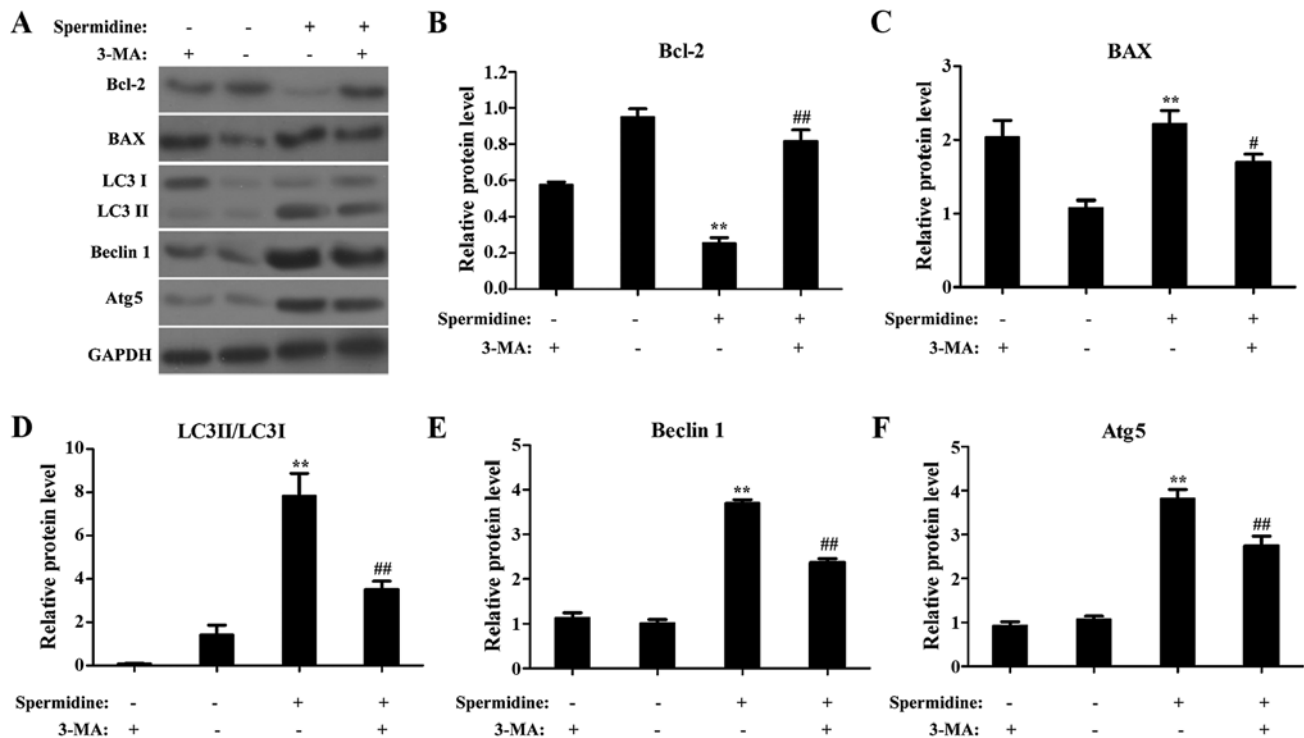
Figure 7. The autophagy inhibitor 3-MA blocks the effects of spermidine in HeLa cells. (A) Western blotting detected LC3 II/LC3 I, Beclin 1, Atg5, BAX, and Bcl-2 in HeLa cells treated with the control, spermidine (120 μM), and/or 3-MA (4 mM). GAPDH was used as the internal control. (B-F) Quantitative analysis of the relative protein expression for (B) Bcl-2, (C) BAX, (D) LC3 II/LC3 I, (E) Beclin 1, and (F) Atg5 as displayed in A (n=3). Data are expressed as the means ± SD. **P<0.01, compared with the controls. ##P<0.01, compared with spermidine treatment. The controls were treated with 0.1% DMSO.

Spermidine induces autophagy of HeLa cells. LC3 acts as a marker of the autophagosome membrane, and is involved in the formation of the autophagic body, and LC3-I/II conversion reflects the number of autophagosomes (23). Beclin 1 is a specific gene associated with autophagy in mammals, which is associated with phospholipid inositol triphosphate-kinase (PI3K), and participates in the formation of autophagosomes (24). Atg5 is a central regulator necessary for autophagy in terms of its involvement in autophagosome elongation (25). Notably, as shown in Fig. 6, western blotting results revealed that LC3 II/LC3 I, Atg5 and Beclin 1 protein levels were significantly increased by spermidine treatment in HeLa cells. These results revealed that spermidine induced autophagy in HeLa cells.

Autophagy mediates the spermidine-induced effects in HeLa cells. To uncover a relationship between apoptosis and autophagy, we used a specific drug inhibitor (3-MA) to block autophagy and analyzed the spermidine-induced effect on HeLa cells. As shown in Fig. 7A, western blots indeed revealed that 3-MA (a specific inhibitor of autophagy) blocked the increase induced by treatment with spermidine in LC3 II/LC3 I, Beclin 1, Atg5, and BAX in HeLa cells (Fig. 7A and C-F), and also blocked the decrease induced by treatment with spermidine in Bcl-2 in HeLa cells (Fig. 7A and B). Moreover, 3-MA significantly blocked the spermidine-induced apoptosis (Fig. 8A-E) and spermidine-inhibited proliferation (Fig. 8F) in HeLa cells. These results provide additional evidence that autophagy mediates spermidine-induced inhibition of cellular proliferation and promotion of apoptosis in HeLa cells.

Discussion

Primary polyamines, putrescine, spermidine and spermine are involved in cellular proliferation, differentiation, tissue development, and carcinogenesis by maintaining chromatin structure, membrane stability and regulating ion channels (14,26). Since 1971, when spermidine was found to be markedly elevated in the urine of many cancer patients (27), there have been attempts to use the biosynthetic pathway of polyamines as therapeutic targets or to use levels of polyamines as prognostic markers for cancer patients. Polyamine depletion has been suggested for cancer prevention, but α-difluoromethylornithine (DFMO) was not clinically effective neither alone nor in combination with other agents (28). Depletion of polyamines by spermidine/spermine N1-acetyltransferase (SSAT) significantly inhibited cellular proliferation, migration, and invasion through the AKT/GSK3β/β-catenin signaling pathway in hepatocellular carcinoma and colorectal cancer cells (29). However, previous studies reported that inhibition of spermidine production promoted colorectal cancer growth (15), and that exogenous spermidine suppressed hepatocellular carcinomas in mice (16). Also, spermidine can alter protein acetylation patterns to regulate autophagy and promote longevity in multiple model systems (30,31). Therefore, the precise biochemical function of spermidine in cancer requires clarification.

In the present study, we chose to assess the effects of spermidine on HeLa cells. Previous studies have revealed that overexpressed SRM (spermidine synthase) in colorectal cancer cell lines CT-26 or MC-38 largely increased spermidine
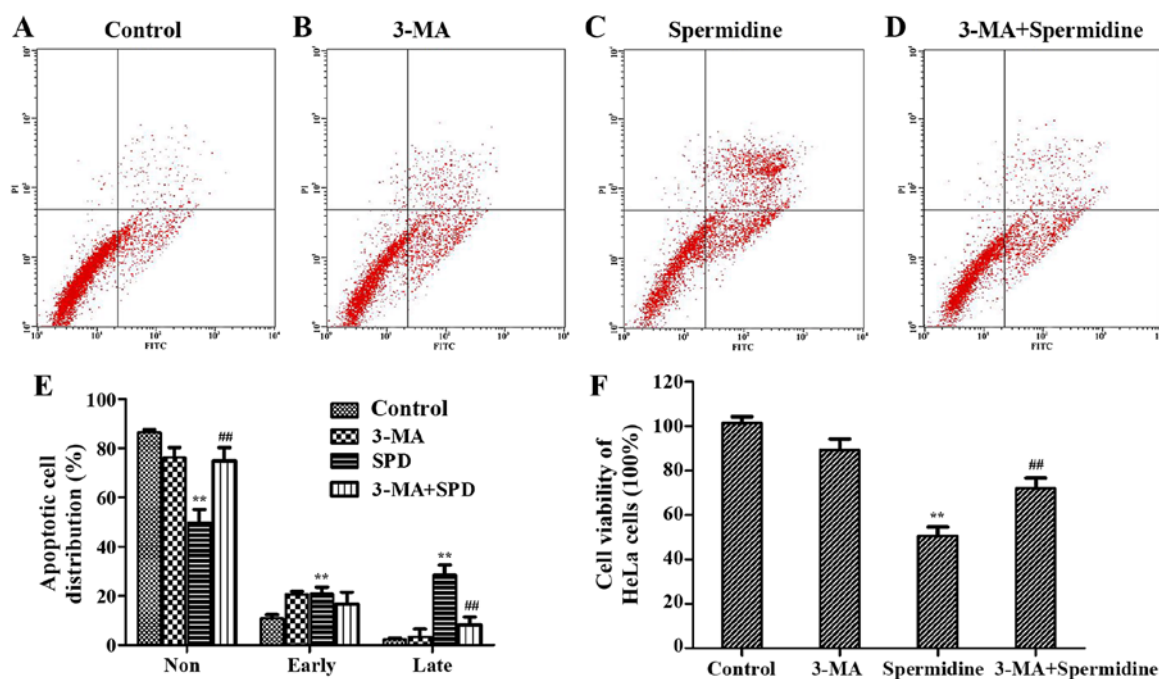
Figure 8. The autophagy inhibitor 3-MA blocks the induction of apoptosis and inhibition of proliferation in HeLa cells with spermidine treatment. (A-D) Flow cytometric analysis of Annexin V-FITC/PI-stained HeLa cells treated with (A) the control medium, (B) 3-MA, (C) spermidine, or (D) spermidine plus 3-MA (n=3). Cells were characterized as healthy cells (bottom left quadrant), necrotic (top left quadrant), early apoptotic (bottom right quadrant), or late apoptotic (top right quadrant). (E) Quantified data regarding the apoptotic rates of HeLa cells as displayed in A-D. (F) CCK-8 assays were performed on HeLa cells after 24 h in control media, or with 3-MA, spermidine, or spermidine plus 3-MA (n=6). Data are expressed as the means ± SD. $^{**}P<0.01$, compared with the controls. $^{##}P<0.01$, compared with spermidine treatment. Controls were treated with 0.1% DMSO.

production; consequently, CT-26- or MC-38-inoculated tumors were inhibited (15). Consistent with these studies, our results demonstrated that spermidine inhibited the proliferation of cervical cancer cells in a dose-dependent manner (Fig. 1). However, we found that the mRNA level of ODC1, SRM and SMOX were significantly decreased, while the mRNA levels of SSAT and SMS were significantly increased in HeLa cells after spermidine treatment (Fig. 2). It suggested that exogenous spermidine inhibited the synthesis of putrescine and spermidine, and promoted the spermine and spermidine consumption. Depletion of the intracellular polyamines inhibits cell growth in HeLa cells. In addition, we found a significant increase in the number of cells at the S phase (Fig. 3D), suggesting that spermidine inhibited the proliferation of HeLa cells by arresting the cell cycle at the S phase.

Previous studies have shown that excessive polyamine accumulation induces cellular apoptosis in ornithine decarboxylase-overproducing L1210 cells (32). Consistent with these studies, we also found that spermidine significantly induced apoptosis of HeLa cells based upon flow cytometric analysis of Annexin V-FITC/PI-stained cells (Fig. 4). BAX migrates to the mitochondrial membrane and inhibits the action of Bcl-2, causing damage to the mitochondrial membrane; this in turn releases cytochrome *c* and leads to apoptosis via the activation of caspase-3 and caspase-7 (33). In our study, western blot analysis indicated that spermidine treatment resulted in attenuated expression of Bcl-2 and increased expression of BAX and cleaved caspase-3 levels in HeLa cells (Fig. 5). Thus, these results support the concept that the enhanced apoptosis of HeLa cells treated with spermidine is due to the activation of mitochondrial apoptotic pathways.

Autophagy is another cellular degradative pathway that is essential for organismal homeostasis, and it has been implicated in protecting organisms from cancer (34,35). Previous studies have revealed that exogenous spermidine suppresses hepatocellular carcinoma in mice by activating MAP1S-mediated autophagy (15). Glaucocalyxin inhibited the proliferation of human cervical cancer cells *in vitro* through the induction of autophagy by the phosphatidylinositol-4,5-bisphosphate 3-kinase/Akt signaling pathway (13). The natural agent HMDB exerted antitumor effects on human cervical cancer cells by induction of autophagy followed by increased expression of LC3 II/LC3 I and Beclin 1 (36). Resveratrol-induced cell death in OVCAR-3 human ovarian cancer cells occured via induced autophagy followed by increased Atg5 expression and promotion of LC3 cleavage (37). Consistent with these studies, we also found that spermidine treatment resulted in increased LC3 II/LC3 I, Atg 5, and Beclin 1 protein levels in HeLa cells (Fig. 6). These results revealed that spermidine inhibited the proliferation of HeLa cells by activating autophagy.

Previous evidence has suggested the existence of crosstalk between the apoptotic and autophagic signaling pathways (38,39). Activation of ROS production simultaneously induced both autophagy and apoptosis in cancer cells (40). Autophagy itself can induce cell death, a process known as autophagic cell death (41). Resveratrol promoted autophagic cell death in chronic myelogenous leukemia cells via JNK-mediated p62/SQSTM1 expression and AMPK activation (42). Beclin 1 is an essential autophagic effector that plays important roles in crosstalk with the apoptotic pathway, interacting with antiapoptotic Bcl-2 proteins (6). Notably, knockdown of

Beclin 1 promoted cell growth and inhibited apoptosis in the A549 human lung cancer cell line (43). Atg5 functions as a switch, shifting the process of cell death from autophagy to apoptosis. Pre-treatment with 3-methyladenine or Atg5-shRNA attenuated justicidin A-induced LC3-II expression and LC3 puncta formation and blocked justicidin A-induced suppression in cell growth via inhibition of apoptosis (44). The present study indicates that spermidine inhibits HeLa cells through both apoptosis and autophagy. We found that the autophagy inhibitor 3-MA attenuated the levels of LC3 II/LC3 I, Atg 5, Beclin 1 and BAX, and enhanced the level of Bcl-2 in HeLa cells treated with spermidine (Fig. 7), and blocked spermidine-induced apoptosis and the spermidine-inhibited proliferation of HeLa cells (Fig. 8). These results strongly demonstrated that autophagy mediates the effects of spermidine on HeLa cells.

In summary, we herein demonstrated that spermidine suppressed proliferation and promoted apoptosis of HeLa cells, and that these effects were mediated by autophagic activation. In our study, the results indicated novel effects of spermidine with respect to its potential use in cervical cancer therapy and provided aspects of the details of the underlying mechanisms of action, focusing on the crosstalk between apoptosis and autophagy.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YC and XW conceived and designed the study. YC, HZ, XC and ZS performed the experiments. YC and XW wrote the paper. YC, XW and HZ reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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