

# Effect of sufentanil on the viability and apoptosis of cervical cancer cells via the inactivation of PI3K/AKT/mTOR signaling pathway

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**Objective:** To investigate the effect of sufentanil on the viability and apoptosis of cervical cancer HeLa cells, and identify the possible molecular mechanism. **Materials and methods:** Cervical cancer HeLa cells were treated with different concentrations of sufentanil at 0, 0.5, 5, 50, and 500 nmol/L, respectively. The cell viability of HeLa cells was detected by CCK-8 and EdU assay. Cell apoptosis of HeLa cells was observed by flow cytometry and Hoechst 33258 staining. The relative expression of PI3K, p-PI3K, AKT, p-AKT, mTOR, and p-mTOR was detected by quantitative real-time PCR and Western blotting analysis. PI3K agonist was applied to activate PI3K/AKT signaling, and its effect on the viability and apoptosis of HeLa cells by 500 nmol/L sufentanil was also determined. **Results:** Sufentanil had a significant inhibitory effect on the viability of HeLa cells, and the inhibition rates increased with the time and concentration. As the concentration of sufentanil increased, the apoptosis rates of HeLa cells increased. Furthermore, sufentanil markedly inhibited the protein expression of p-PI3K, p-AKT, and p-mTOR in a concentration-dependent fashion, but had no effect on the PI3K, AKT, and mTOR mRNA and protein expression. In addition, PI3K agonist significantly increased p-PI3K, p-AKT, and p-mTOR protein expression in HeLa cells, and could partly reverse the effect of 500 nmol/L sufentanil on cell viability and apoptosis. **Conclusion:** Sufentanil has an inhibitory effect on the viability of cervical cancer HeLa cells by inducing cell apoptosis, the mechanism may be achieved through the inactivation of PI3K/AKT/mTOR signaling pathway.

## Keywords

Sufentanil; Effect; HeLa; PI3K/AKT/mTOR; Mechanism

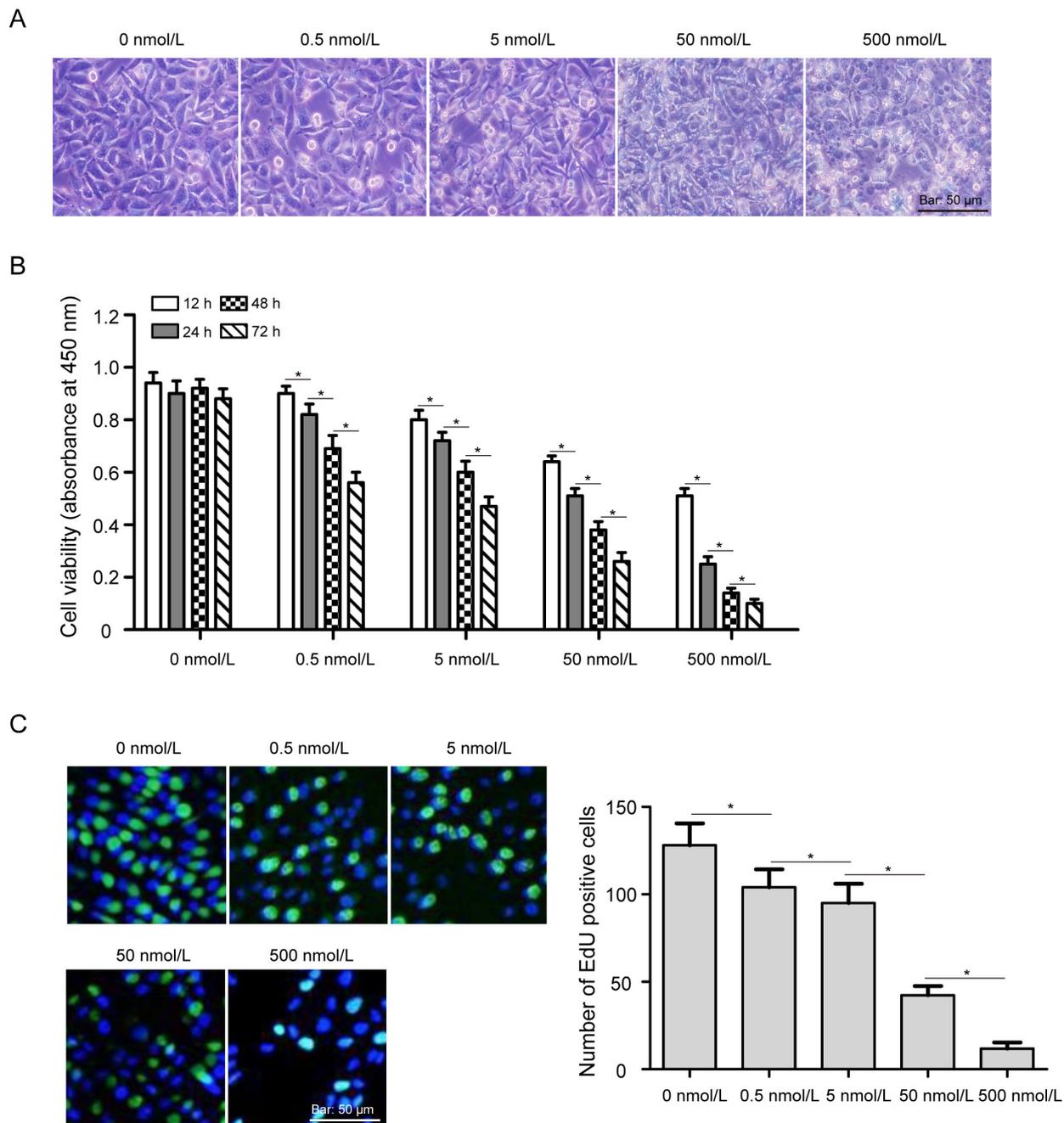
## 1. Introduction

Cervical cancer is one of the common gynecological malignancies worldwide [1]. Among malignant tumors of the female reproductive system, the incidence of cervical cancer is close to breast cancer. The cause of cervical cancer is clear, and the technology for screening, early diagnosis and treatment are mature, and there are a variety of therapy options for cervical cancer treatment in different regions. In developed countries, the morbidity and mortality of cervical cancer are reduced with the development of an organized cervical

cancer screening program based on cytology and human papilloma virus (HPV) examination [2, 3]. In recent decades, with the progress of etiological research, the development and application of HPV preventive vaccines have made it possible to prevent cervical cancer progression [4, 5]. At present, operation, chemotherapy and radiotherapy are the most commonly used treatments for cervical cancer. However, due to drug resistance of cervical cancer cells against the therapeutic agents, its treatment still faces greater challenges [6, 7].

Sufentanil (molecular formula:  $C_{22}H_{30}N_2O_2S$ , molecular weight: 386.55) is a synthetic opioid receptor agonist that is used as a primary anesthetic agent [8]. It is a potent narcotic analgesic with similar pharmacological effect to morphine. Sufentanil is administered by the intravenous, epidural and sublingual routes, and is widely used in clinical anesthesia, perioperative analgesia and cancer pain treatment [9]. Clinical trial has shown that its analgesic strength is about 100 times that of morphine, its analgesic effect is fast, and the side effects are smaller than morphine [10]. Studies have shown that sufentanil regulates the expression of various factors in the PI3K/AKT signaling to protect the myocardium from ischemia-reperfusion [11, 12]. However, there is currently a lack of research on the survival effect of sufentanil on cervical cancer cells.

Therefore, this study aims to explore the potential mechanism of action by detecting the effect of different concentrations of sufentanil at 0, 0.5, 5, 50, and 500 nmol/L on the viability and apoptosis of HeLa cells. Our data found that sufentanil has an inhibitory effect on the viability of cervical cancer HeLa cells by inducing cell apoptosis via the inactivation of PI3K/AKT/mTOR signaling pathway. The research will provide a theoretical basis for finding effective biological targets and realizing precise treatment of cervical cancer.



**Fig. 1. The effect of sufentanil on the viability of HeLa cells.** (A) Images of HeLa cells treated with 0, 0.5, 5, 50, and 500 nmol/L of sufentanil for 72 h. Bar: 50  $\mu$ m. (B) CCK-8 assay analysis of cell viability of HeLa cells, and found the viability of HeLa cells was significantly decreased by sufentanil in a concentration-dependent and time-dependent fashion. (C) Representative images for EdU assay, and results showed that the number of EdU positive cells was decreased by sufentanil treatment. Each bar represents the mean  $\pm$  SD for triplicate experiments. \* $P < 0.05$ .

## 2. Material and methods

### 2.1 Reagents

The Cell Counting Kit-8 (CCK-8) kit, Hoechst 33258 staining kit, dimethyl sulfoxide (DMSO), trypsin, penicillin, and streptomycin were provided by Sigma company (St Louis, MO, USA). sufentanil was purchased from Yichang Humanwell Pharmaceutical Co., Ltd (Yichang, Hubei, China). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were provided by Thermo

Fisher Scientific Co., Ltd (Foster City, CA, USA). Annexin V-propidium iodide (PI) staining reagent was bought from BD Biosciences company (Foster City, CA, USA). PI3K agonist (insulin-like growth factor-1) [13] and agonist control were obtained from Biocode Hyclon company (Brussels, Belgium, France).

## 2.2 Cell line and cell cultivation

Cervical cancer HeLa cell line was bought from the China Centre for Type Culture Collection (CCTCC, Wuhan, China). Cell cultivation was conducted in DMEM medium, added 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. The environmental setting was 37 °C with 5% CO<sub>2</sub>, with a replacement of the cultivation medium every two days.

## 2.3 Quantitative real-time PCR

Total RNA of each sample was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instruction. Total RNA in the amount of 1 µg was reversely transcribed to cDNA using a First-strand cDNA Synthesis Kit (New England Biolabs, Ipswich, MA, USA) according to manufacturer's protocol. Then, the cDNA was used as a PCR template to detect PI3K, AKT, and mTOR expression by a SYBR PremixEx Taq II kit (TaKaRa, Beijing, China) on the Agilent AriaMx Real-time PCR System (Agilent, Santa Clara, CA, USA), according to manufacturer's instruction. GAPDH was used as a reference gene for normalization. The primer sequences were shown in Table 1. The following PCR program was conducted: denaturation at 98 °C for 10 min, followed by 40 cycles consisting of denaturation at 95 °C for 15 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s. The relative expression level of each gene was calculated using ( $2^{-\Delta\Delta Ct}$ ) method.

**Table 1. The primer sequences used for quantitative real-time PCR.**

Gene	Sequences
PI3K	Forward: 5'-CCACGACCATCATCAGGTGAA-3'
	Reverse: 5'-CCTCACGGAGGCATTCTAAAGT-3'
AKT	Forward: 5'-TCCTCCTCAAGAATGATGGCA-3'
	Reverse: 5'-GTGCGTTTCGATGACAGTGGT-3'
mTOR	Forward: 5'-ATGCTTGAACCGGACCTG-3'
	Reverse: 5'-TCTTGACTCATCTCTCGGAGTT-3'
GAPDH	Forward: 5'-AGCCACATCGCTCAGACAC-3'
	Reverse: 5'-GCCCAATACGACCAAATCC-3'

## 2.4 Western blotting analysis

Total protein of each sample was lysed and extracted by RIPA buffer (Beyotime Biotechnology, Shanghai, China). The concentration of protein was determined with a BCA protein kit (Sigma, St Louis, MO, USA). Then 30 µg protein from each sample lysates was electrophoresed by 10% SDS-PAGE (Beyotime, Shanghai, China; P0012A), followed by transferring into polyvinylidene fluoride membranes (PVDF; Beyotime Biotechnology). The membranes were blocked by 5% skimmed milk for 2 h at 37 °C, and incubated in the primary antibodies which consist of anti-phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) antibody (mouse, monoclonal, 1 : 1000, ab135253, Abcam, Cambridge, MA, USA), anti-p-PI3K (phospho Y607) antibody (rabbit, polyclonal, 1 : 400, ab182651, Abcam), anti-AKT serine/threonine kinase

(AKT) antibody (rabbit, polyclonal, ab8805, 1 : 500, Abcam), anti-p-AKT (phospho T308) antibody (rabbit, polyclonal, 1 : 200, ab38449, Abcam), anti-mechanistic target of rapamycin kinase (mTOR) antibody (rabbit, polyclonal, 1 : 500, ab2732, Abcam), anti-p-mTOR (phospho S2448) antibody (rabbit, polyclonal, 1 : 500, ab84400, Abcam), and anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (mouse, monoclonal, 1 : 5000, ab8245, Abcam) at 4 °C overnight. The membranes were subsequently incubated in the secondary horseradish peroxidase (HRP)-combined antibodies: anti-mouse IgG H&L (HRP) (goat, 1 : 2500, ab205719, Abcam) and anti-rabbit IgG H&L (HRP) (goat, 1 : 2500, ab205718, Abcam) and for 1 h at 37 °C, and washed with tris-buffer saline tween (TBST) for three times. The protein bands were analyzed by an enhanced chemiluminescence (ECL) kit (Millipore, Billerica, MA, USA), according to manufacturer's instruction. GAPDH was used as internal reference. Grey values of the strips were calculated by ImageJ software (version 5.0; Bio-Rad, Hercules, CA, USA).

## 2.5 CCK-8 assay

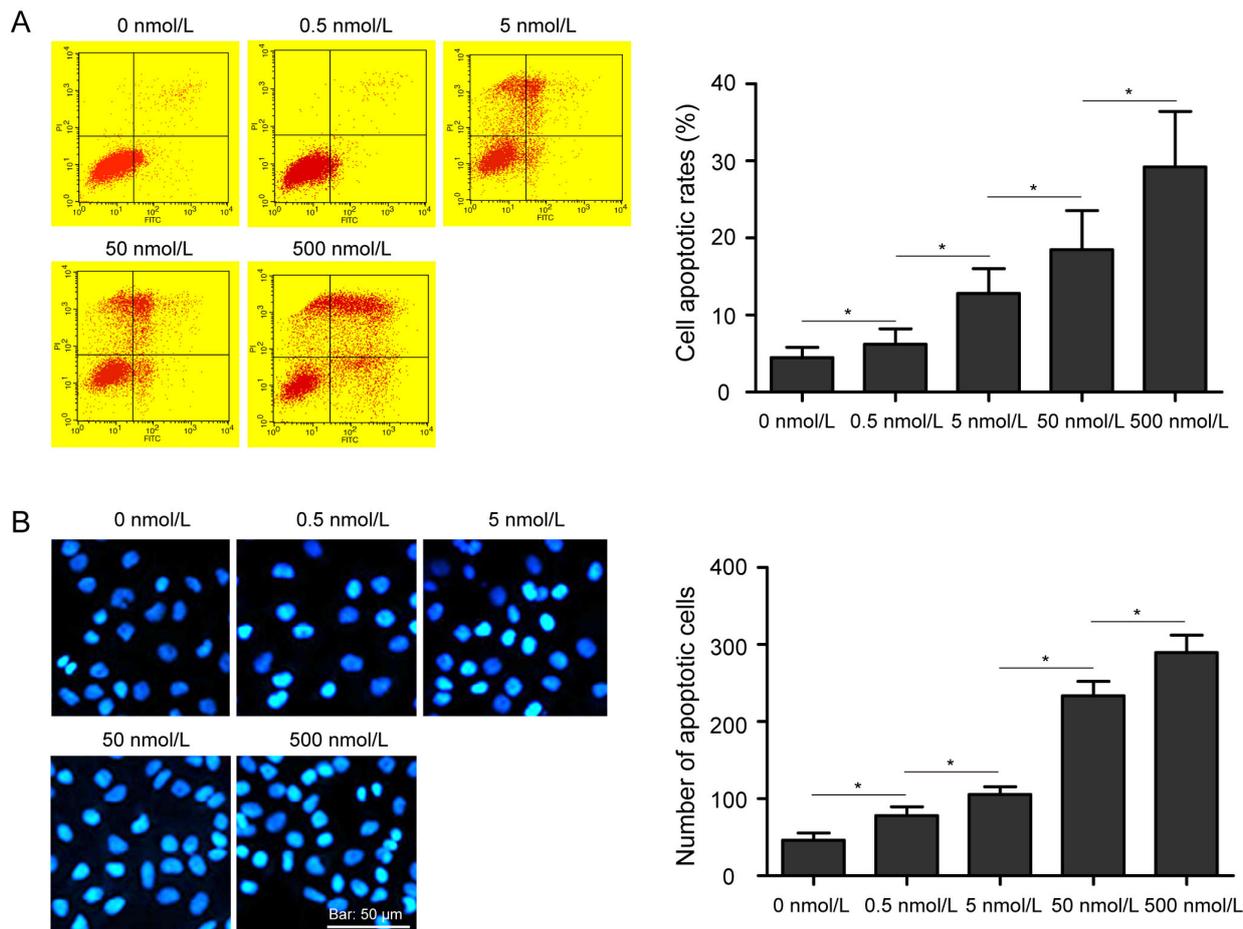
HeLa cells were implanted at  $8 \times 10^3$  cells/well in 96-well plates. Following 24 h culturing, the cells were divided into four processing time groups for 12 h, 24 h, 48 h and 72 h, then exposed to five different levels of sufentanil at concentrations of 0, 0.5, 5, 50, and 500 nmol/L, respectively [14]. Subsequently, the CCK-8 assay was applied to test the viability capability of cells. Every well-received 10 µL of CCK-8 solution apart from 90 µL cultivation medium and maintained at 37 °C for 3 h. The absorbance at 450 nm was tested and was in direct proportion to the cell viability.

## 2.6 EdU assay

For EdU cell proliferation assay,  $5 \times 10^4$  cells were placed in each well of the 24-well plate overnight. Cells were then added to different levels of sufentanil at concentrations of 0, 0.5, 5, 50, and 500 nmol/L, respectively for 48 h cell culture. The adherent cells were washed with PBS for three times, and the proliferation of the cells was detected by a 5-ethynyl-20-deoxyuridine (EdU) assay kit (Ribobio, Guangzhou, China), according to the manufacturer's instruction. The images of stained cells were conducted for photographing and counting under a BX51 optical microscope (Olympus, Japan).

## 2.7 Hoechst 33258 staining

HeLa cells were put into 6 well plates at a density of  $4 \times 10^6$  cells/well with 2 ml of DMEM and FBS for culturing overnight, the cells were treated with 0, 0.5, 5, 50, and 500 nmol/L concentrations of sufentanil for 48 h, respectively. After collection, the cells were fixed with 4% paraformaldehyde at 37 °C for 2 h, were stained with Hoechst 33258 for 5 min at 37 °C, according to the instruction. The observation of nuclei was conducted in each condition under a Zeiss Axiovert 200 fluorescence microscope (Zeiss, Heidenheim, Germany).



**Fig. 2. The impact of sufentanil on the apoptosis of HeLa cells.** (A) Representative images of flow cytometry via annexin V-FITC/ PI double staining following treatment of HeLa cells with 0, 0.5, 5, 50, and 500 nmol/L concentrations of sufentanil for 48 h. (B) HeLa cells were treated by sufentanil for 48 h and then observed via Hoechst 33258 staining. Bar: 50  $\mu$ m. Each bar represents the mean  $\pm$  SD for triplicate experiments. \* $P$  < 0.05.

### 2.8 Flow cytometry

Annexin V-PI staining followed by flow cytometry was performed for the cell apoptosis analysis.  $4 \times 10^6$  cells/well HeLa cells were cultured in cell 6-well plates and treatment with sufentanil at concentrations of 0, 0.5, 5, 50, and 500 nmol/L, respectively. After 48 h culture, cells were collected by trypsin (without EDTA) digestion, cold PBS wash for three times and centrifugation at 1200 g for 10 min.  $1 \times$  binding buffer was then added for cell suspension, followed by 3  $\mu$ L of Annexin V-FITC staining for 15 min and 2  $\mu$ L of PI staining for 20 min at 37  $^{\circ}$ C, and cell apoptosis was observed by a flow cytometer (BD Biosciences).

### 2.9 Rescue assays

HeLa cells were implanted at 6-well and 96-well plates. Following 24-h culturing, cells were treated with 500 nmol/L sufentanil and PI3K agonist or agonist control (0.5  $\mu$ g for 96-well, 2  $\mu$ g for 6-well). After 48 h cell culture, the cells were collected and CCK-8 assay and Flow cytometry were performed to determine cell viability and apoptosis.

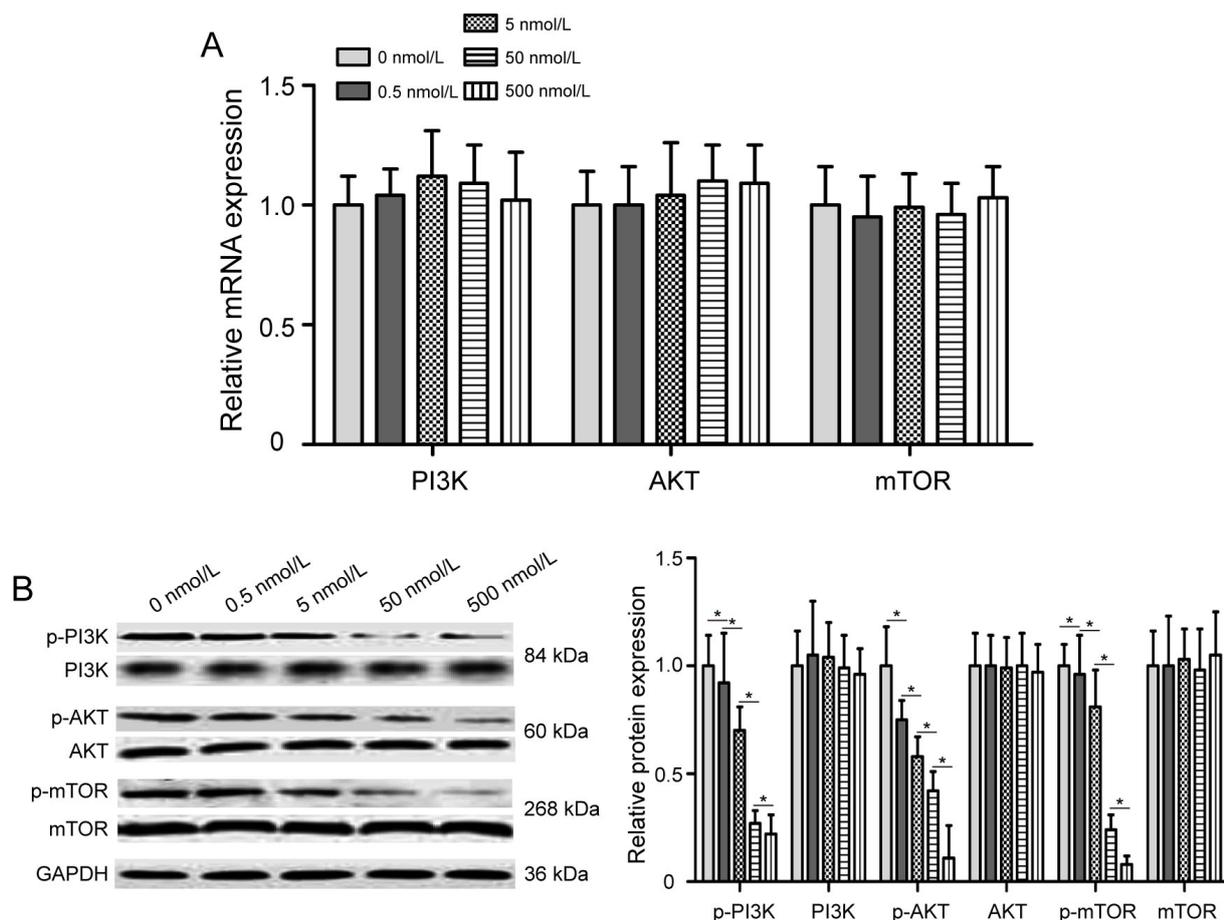
### 2.10 Statistical analysis

Data were processed by SPSS17.0 statistical software (SPSS, Inc., Chicago, USA) and presented as the mean  $\pm$  standard deviation (SD) of results from at least three independent experiments. A Student t-test was used for statistical comparison between means in the two groups. Differences among more than two groups in the above assays were assessed by one-way ANOVA followed by Dunnett's post hoc test.  $P$  < 0.05 was considered statistically significant.

## 3. Results

### 3.1 Sufentanil inhibits HeLa cell viability in vitro

Different concentrations of sufentanil (0, 0.5, 5, 50, and 500 nmol/L) were applied in the treatment on HeLa cells for 72 h cell culture to identify the effect of sufentanil on cell viability. The images of HeLa cells in the five different concentration groups were shown in Fig. 1A, HeLa cells after treatment with 0.5, 5, 50, and 500 nmol/L of sufentanil became rounded and were floating in DMEM, with lower adhesive cells than the 0 nmol/L of sufentanil group. The CCK-8 assay revealed that the viability of HeLa cells was significantly decreased by sufentanil in a concentration-dependent and time-



**Fig. 3. Sufentanil suppresses the activation of PI3K/AKT/mTOR signaling pathway.** (A) Quantitative real-time PCR analysis of mRNA expression of PI3K, AKT, and mTOR in HeLa cells after treatment of sufentanil. GAPDH was used as internal reference. (B) Western blotting analysis the protein levels of PI3K, p-PI3K, AKT, p-AKT, mTOR, and p-mTOR in HeLa cells by treating sufentanil. Each bar represents the mean  $\pm$  SD for triplicate experiments. \* $P < 0.05$ .

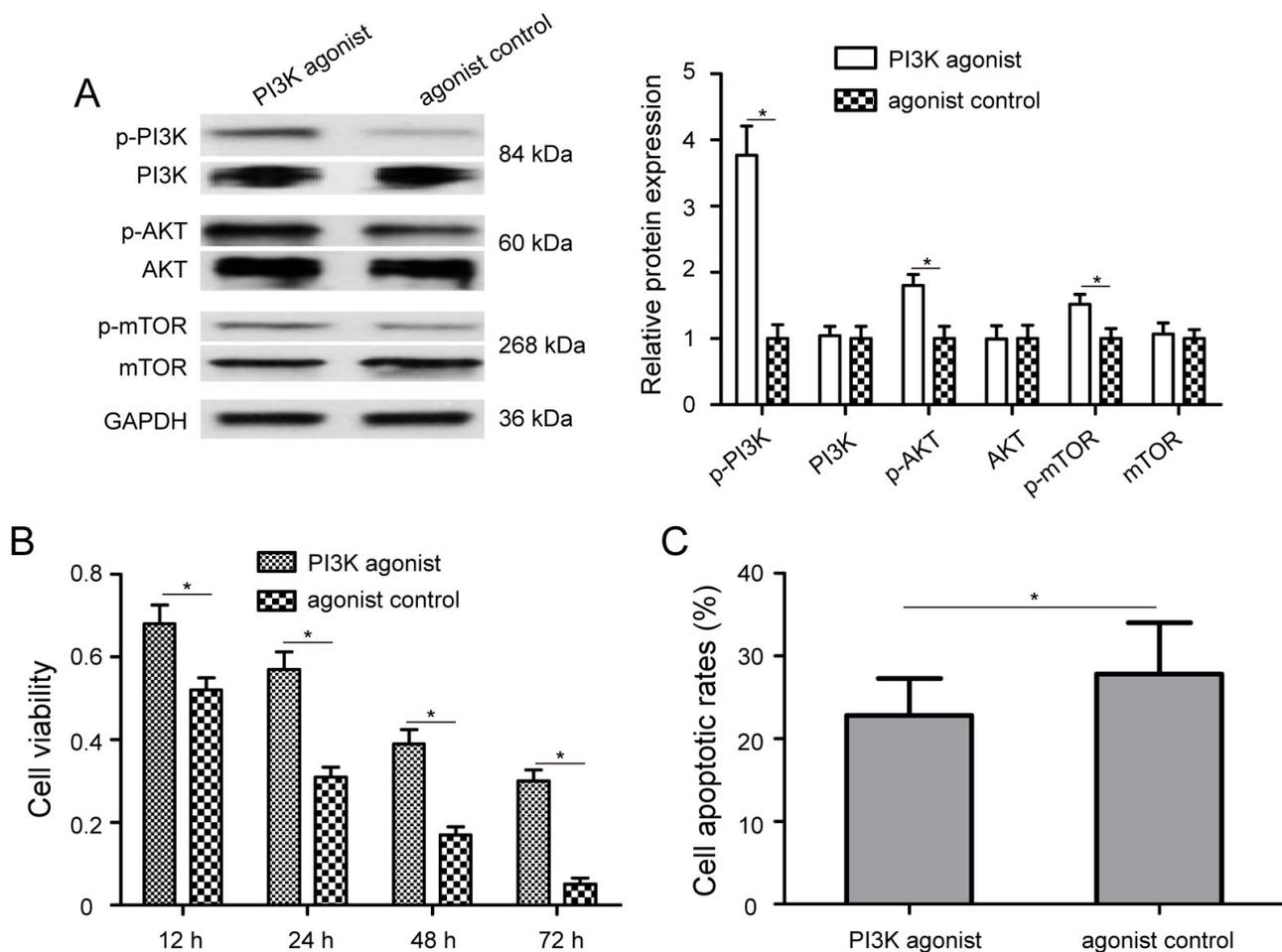
dependent fashion (Fig. 1B, all  $P < 0.05$ ). To further confirm the potential inhibitory action of sufentanil, a EdU cell proliferation assay was conducted. As illustrated in Fig. 1C, the number of EdU positive cells was dropped by sufentanil in a dose-dependent manner (all  $P < 0.05$ ). These results revealed that sufentanil could inhibit the viability of HeLa cells *in vitro*.

### 3.2 Sufentanil induces HeLa cell apoptosis *in vitro*

After being treated with sufentanil at the concentrations of 0, 0.5, 5, 50, and 500 nmol/L, the cell apoptosis of HeLa cells was determined by flow cytometry. According to the results in Fig. 2A, the cell apoptotic rates of HeLa cells were elevated with the increase on the dosage of sufentanil (all  $P < 0.05$ ). Subsequently, we performed Hoechst 33258 staining to observe of nuclei after being treated with sufentanil. It was found that the number of apoptotic cells of HeLa cells was upregulated with the increase on the dosage of sufentanil (Fig. 2B, all  $P < 0.05$ ), suggesting that sufentanil could induce the apoptosis of HeLa cells *in vitro*.

### 3.3 Sufentanil suppresses the activation of PI3K/AKT/mTOR signaling pathway of HeLa cells

Furthermore, in order to investigate whether sufentanil regulated PI3K/AKT/mTOR signaling pathway activation, the relative mRNA expression of PI3K, AKT, and mTOR was detected by quantitative real-time PCR. The results of Fig. 3A showed that the mRNA levels of PI3K, AKT, and mTOR in HeLa cells demonstrated no significant difference among the five different concentration groups. In addition, Western blotting analysis were performed to measure the protein levels of PI3K, p-PI3K, AKT, p-AKT, mTOR, and p-mTOR in HeLa cells. Similarly, there was no significant difference in the protein levels of PI3K, AKT, and mTOR in the five different concentration groups. At the same time, the data observed that the p-PI3K, p-AKT, and p-mTOR levels were downregulated in a concentration-dependent fashion (Fig. 3B, all  $P < 0.05$ ). These results suggested that sufentanil could suppress the activation of PI3K/AKT/mTOR signaling pathway in HeLa cells.



**Fig. 4. PI3K agonist reverses the effect of sufentanil on the viability and apoptosis of HeLa cells.** (A) The PI3K/AKT/mTOR signaling pathway was activated in HeLa cells by PI3K agonist, as confirmed by Western blotting analysis. (B) PI3K agonist reversed the viability-suppressive effect of sufentanil on HeLa cells. (C) The sufentanil mediated promotive effect on HeLa cell apoptosis was partially reversed by treatment with PI3K agonist. Each bar represents the mean  $\pm$  SD for triplicate experiments. \* $P < 0.05$ .

### 3.4 PI3K agonist reverses the effects of sufentanil on the viability and apoptosis of HeLa cells in vitro

Due to the regulation of sufentanil on PI3K/AKT/mTOR signaling pathway in HeLa cells, we speculated that the role of sufentanil in regulating cell viability and apoptosis was mediated by this signaling. We then transfected PI3K agonist and agonist control into the 500 nmol/L of sufentanil treated HeLa cells, and functional rescue experiments were performed. It was discovered in the PI3K agonist group of HeLa cells that the p-PI3K, p-AKT, and p-mTOR levels were increased compared with the agonist control group (Fig. 4A, all  $P < 0.05$ ), but the protein levels of PI3K, AKT, and mTOR had no significant difference, indicating that the PI3K/AKT/mTOR signaling pathway is successfully activated in HeLa cells by PI3K agonist. Notably, after PI3K agonist treatment, the viability of HeLa cells by 500 nmol/L of sufentanil was evidently increased compared with the agonist control group (Fig. 4B, all  $P < 0.05$ ). Moreover, we found that 500 nmol/L of sufentanil mediated promotive effect on HeLa cell apoptosis were partially reversed by treat-

ment with PI3K agonist (Fig. 4C,  $P < 0.05$ ). Collectively, these results demonstrated that the effect of sufentanil on the viability and apoptosis of HeLa cells is mediated by the inactivation of PI3K/AKT/mTOR signaling pathway.

## 4. Discussion

In recent years, there have been a large number of studies supported that some anesthetic agents can achieve the purpose of inhibiting tumor development by decreasing cell viability and promoting cell apoptosis [15]. For example, propofol activates the response to hypoxia in pancreatic carcinoma by targeting ADAM metallopeptidase domain 8 expression, and achieves great value for therapeutic effects [16]. Sevoflurane inhibits the progression of ovarian carcinoma via downregulating stanniocalcin 1, suggesting sevoflurane may serve as a potential anti-cancer agent in ovarian carcinoma therapy [17]. Lidocaine suppresses the proliferation of lung carcinoma by regulating the expression of Golgi transport 1 homolog A [18]. In addition, ropivacaine represses the migration of esophageal carcinoma via sodium-

channel-independent but prenylation-dependent inhibition of Rac1/JNK/paxillin/FAK signaling pathway [19]. Sufentanil is a short acting synthetic opioid analgesic most commonly used for pain relief during medical operations [8]. The present study aims to figure out the role of sufentanil on HeLa cells, and explore its underlying mechanism, making it possible for a promising alternative therapeutic agent for patients with cervical cancer. The results of this study showed that sufentanil had an inhibitory effect on the viability of cervical cancer HeLa cells by inducing cell apoptosis via the inactivation of PI3K/AKT/mTOR signaling pathway.

Modern medical research has been shown that sufentanil has a wide range of physiological and pathological activities in pharmacology [20]. Sufentanil has been found to reduce hepatic ischemia-reperfusion injury through suppressing the p38/ERK/JNK/NF-kappaB-p65/COX2 signaling pathway [21]. Also, in Zhang *et al.*'s study, sufentanil is shown to attenuate oxaliplatin cytotoxicity of colorectal carcinoma via inhibiting connexin 43-composed gap junction function [22]. Wu *et al.* demonstrated that sufentanil can inhibit the cell viability and induce the apoptosis of gastric carcinoma SGC-7901 cells *in vitro* [14]. Recently Jiang *et al.*'s study has reported that sufentanil impairs autophagic degradation and inhibits cell migration in human large cell lung cancer cell line NCI-H460 *in vitro* [23]. However, the effect of sufentanil on viability and apoptosis in HeLa cells and its mechanism remain still uncertain. Hence, we performed 0, 0.5, 5, 50, and 500 nmol/L of sufentanil to stimulate HeLa cells, and found that the viability of HeLa cells was significantly decreased by sufentanil in a concentration-dependent and time-dependent fashion. At the same time, we observed that the cell apoptosis of HeLa cells was elevated in a drug-dependent manner. The results were further confirmed by EdU cell proliferation assay and Hoechst 33258 staining, suggesting sufentanil treatment inhibits cell viability but promotes cell apoptosis of HeLa cells *in vitro*.

More and more studies have confirmed the key role of PI3K/AKT/mTOR signaling pathway in the maintenance of cancer development [24, 25]. Wu *et al.* found that silencing of glucose transporter type 1 gene inhibits proliferation and promotes apoptosis of colorectal carcinoma by inactivating PI3K/AKT/mTOR signaling [26]. Zhao *et al.* demonstrated that metformin suppresses the proliferation of human endometrial carcinoma by inhibiting PI3K/AKT/mTOR signaling [27]. Activation of the PI3K/AKT/mTOR pathway in breast carcinoma stem-like cells is required for colony-formation ability *in vitro* and tumorigenicity *in vivo* [28, 29]. In our study, we discovered that sufentanil inhibited the protein expression of p-PI3K, p-AKT, and p-mTOR in a concentration-dependent fashion, suggesting sufentanil may induce the inactivation of PI3K/AKT/mTOR signaling. These above results encouraged us to hypothesize that sufentanil might exert its role by regulating PI3K/AKT/mTOR pathway. In support of this hypothesis, increased p-PI3K, p-AKT, and p-mTOR expression by PI3K agonist treatment

was noted in 500 nmol/L of sufentanil treated HeLa cells. Rescue experiments provided the strong evidences that PI3K agonist could partly reverse the effect of 500 nmol/L sufentanil on cell viability and apoptosis of HeLa cells. These data demonstrated that the effect of sufentanil on the viability and apoptosis of HeLa cells is mediated by the inactivation of PI3K/AKT/mTOR signaling pathway.

However, due to the limitation of manpower and material resources, there're still some limitations in our study. First, the role of sufentanil on others gynecologic malignancies *in vitro* was absence. Second, the effect of sufentanil on inhibition of cervical cancer tumorigenicity and progression *in vivo* was unclear. Third, the other signalling pathway remains to be explored in future studies. Fourth, a multicenter prospective clinical study is required to further confirm support our findings.

## 5. Conclusions

Our study provides supporting evidence that sufentanil induces the the inactivation of PI3K/AKT/mTOR signaling pathway, resulting in the suppression of cell viability as well as the promotion of cell apoptosis in HeLa cells *in vitro*.

## Author contributions

KCL designed the research study. YJ performed the research. YJ and LS analyzed the data. YJ and KL wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

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## Conflict of interest

The authors declare no competing interests.

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