Ropivacaine enhanced the cisplatin-sensitivity of Human colorectal cancer cells by accelerating ROS-mediated apoptosis and ferroptosis

Zhen Zhang
Hubei University of Medicine

Lian Zeng
Huazhong University of Science and Technology

WenBo Zhao
Hubei University of Medicine

Qiang Zhao
Hubei University of Medicine

Qingsong Wang
Hubei University of Medicine

Xudong Ding
Hubei University of Medicine

Qianqian Xu
Hubei University of Medicine

Yu Zhang
Hubei University of Medicine

Pengchao Hu
Hubei University of Medicine

Huiyu Luo (✉ 603983267@qq.com)
Hubei University of Medicine

Research Article

Keywords: Human colorectal cancer cells (LOVO, LOVO/DDP), Cisplatin resistance, Ropivacaine, Apoptosis, Ferroptosis

Posted Date: February 17th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-2590316/v1

License: ☑️ This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

The failure of cisplatin treatment to human colorectal cancer due to cisplatin-sensitivity reduced, it’s necessary to find the new adjuvant agents to increase the cisplatin-sensitivity. Ropivacaine has been demonstrated to inhibit the proliferation and migration of variety of cancer cells, however, it remains unclear whether it could increase the cisplatin sensitivity of colorectal cancer cells. The cisplatin-resistant cell line for colorectal cancer named LOVO/DDP cells were established by repeated induction of different concentration of cisplatin, the cell viability, proliferation, migration, and apoptosis were assessed by Colony-forming assay, Transwell assay, Wound Healing and Annexin V/PI flow cytometry; JC-1 and ROS detection kits were used to determine the levels of mitochondrial membrane potential and reactive oxygen species (ROS). The expression of MMP-9, Nrf-2, GPX4, SLC7A11, SIRT1 proteins were detected by Western-blot and immunofluorescence. Compared with the control group, the activity of LOVO cells decreased significantly and that in LOVO/DDP cells decreased slightly with the increase of cisplatin. After the treatment of ropivacaine combined with cisplatin, the LOVO/DDP cells viability was significantly decreased than cisplatin alone (P<0.01). Compared with cisplatin alone treatment group, the proliferation and migration of LOVO cells and LOVO/DDP cells were significantly reduced in combined group. Ropivacaine combined with cisplatin enhanced apoptosis, the production of ROS, induced mitochondrial dysfunction, and down-regulated anti-ferroptosis and SIRT1 proteins. Those above results demonstrated that ropivacaine could increase the chemosensitivity of cisplatin-resistant human colon cancer cells, and its mechanism may be through promoting cancer cell apoptosis and ferroptosis.

Introduction

With the development of therapeutic strategies to colorectal cancer (CRC), the mortality rate has been declined [1]. Although surgery is the major choice for CRC treatment, the chemotherapy coupled with surgery improves the survival of patients with intermediate and advanced cancer, and cisplatin is widely used as a first-line chemotherapy drug for CRC treatment [2, 3]. Recently, the treatment failure and tumor recurrence are largely due to decreased sensitivity of CRC to cisplatin, there is a significant change of drug resistance and considerable side effects, which present a remarkable limitation for the clinical application of cisplatin [4]. So that it’s necessary to find the new adjuvant agents to increase the cisplatin-sensitivity for CRC.

Ropivacaine is an amide-linked local anesthetic for regional anesthesia and pain management including acute pain, chronic pain, and cancer pain. In addition to hindering the conduction of nerve excitation, ropivacaine has been showed to have a significant anti-tumor effect by many studies [5-7]. Shen et al. reported that ropivacaine significantly inhibited lung cancer cell malignancy by downregulating cell-survival-associated cellular molecules [8]. Wang et al. confirmed ropivacaine inhibited the proliferation and migration of colon cancer by targeting the expression of ITGB1 protein and affecting the activation of its downstream signaling pathways [9]. However, the role of ropivacaine in cisplatin-resistant CRC remains not fully understood. In current study, we mainly explored the ropivacaine function in cisplatin-
resistant CRC and demonstrated that ropivacaine enhanced the cisplatin-sensitivity of Human colorectal cancer cells by accelerating ROS-mediated apoptosis and ferroptosis.

Cisplatin is a platinum-based chemotherapy agent that impairs the DNA repair process by crosslinking with purine bases on DNA, resulting in apoptosis in cancer cells [10, 11]. Therefore, enhancing the anti-apoptotic effect of cisplatin is considered a potential strategy to combat cisplatin-resistance. Ropivacaine has been showed to promote the apoptosis of numerous cancer cells including breast cancer, hepatocellular carcinoma cancer, and colon cancer [12-15]. Our findings are consistent with above, ropivacaine combined with cisplatin significantly increase apoptosis in cisplatin-sensitive and cisplatin-resistant colon cancer cells when compared to cisplatin alone treatment. At present, ferroptosis was defined as a new form of cell death characterized by iron-dependent lipid peroxidation, which has been involved in cisplatin resistance in many cancers [16, 17]. Cancer cells can adapt to an oxidative environment to control ferroptosis, which could reshape tumor niches to promote tumor growth and drug resistance. Therefore, induction of ferroptosis to colon cancer cells alleviates cisplatin resistance [18, 19]. Our findings confirmed that ropivacaine combined with cisplatin significantly induce ferroptosis in cisplatin-sensitive and cisplatin-resistant CRC cells when compared to cisplatin alone treatment. Mitochondrial dysfunction is a common feature of apoptosis and ferroptosis [20], ropivacaine enhanced cisplatin sensitivity of colon cancer cells by the induction of mitochondrial dysfunction. To explore the molecular mechanism of this, through the small molecule docking, we found that ropivacaine could target binding to silencing information regulator 2 related enzyme 1 (SIRT1), an NAD+-dependent deacetylase, which has been to be important for the turnover of defective mitochondria by mitophagy [21]. Ropivacaine downregulated the expression of SIRT1, thereby inducing mitochondrial dysfunction in colon cancer cells, promoting the production of ROS, leading to the apoptosis and ferroptosis. Which may the possible mechanism of ropivacaine enhanced cisplatin sensitivity of colon cancer cells.

In this study, we investigated that the effect of ropivacaine on cisplatin-relevant CRC cells and confirmed ropivacaine enhanced cisplatin sensitivity by prompting the synthesis of ROS, mitochondrial dysfunction, apoptosis, and ferroptosis, which is closely related the inhibition of SIRT1 expression. This finding might provide a new treatment strategy for cisplatin resistance in colon cancer.

**Materials And Methods**

The human colorectal cancer LOVO cells were brought by Procell (Wuhan, Hubei); ropivacaine and cisplatin were from the Selleck Cham (USA), the primary antibodies of Bax, Bcl-2 and Cleaved-Caspase-3, MMP-9 and GAPDH were from the proteintechTM (Wuhan, China); and Nrf-2, GPX4, SLC7A11 were from Cell Signaling Technology (USA). The H₂DCFDA reactive oxygen species (ROS) and mitochondrial membrane potential test kits (JC-1) were from KeyGEN BioTECH (Jiangsu, China); Dylight Fluor® 488 and Dylight Fluor® 594 labeled goat anti-rabbit IgG (H + L) secondary antibodies were from Jackson Immuno Research (West Grove, PA, USA). The MitoTracker Red CMXRos, AnnexinV-FITC/PI apoptosis
detection kit and Hoechst 33258 were from the Beyotime Biotechnology (Shanghai, China), The Fe^{2+} indicator of FerroOrange was from DOJINDO (Japan).

**Establishing cisplatin-resistant cells of LOVO/DDP**

The human colorectal cancer LOVO cells were treated with 1 μg/mL cisplatin for 24h to release the dead cells, then they were cultured in RPMI 1640 medium without cisplatin for proliferation, the process was repeated for five times. Subsequently, the living cells were treated with 2 μg/mL cisplatin as above until the concentration of cisplatin increase to 5 μg/ml. The cisplatin-resistant cells were named LOVO/DDP cells which could stable growth under 5 μg/mL cisplatin treatment.

**Cell Viability**

The sensitivity of LOVO and LOVO/DDP cells to cisplatin was evaluated by the cell counting kit-8 (CCK-8) according to the manufacturer's instruction. Those above cells were separately seeded in the 96-well plate at a density of 2 × 10^3 cells/well and then treated with PBS or various concentrations of cisplatin (1-5 μM) in 100 μl of medium for 48 h, 10 μl of CCK-8 was added to each plate, after co-incubation for 2 h at 37 °C, The absorbance was measured in a microplate reader (SpectraMax®iD3, Molecular Devices, USA) at a wave-length of 450 nm. To assess the anti-tumor effect of ropivacaine, the LOVO and LOVO/DDP cells were treated with ropivacaine alone or combination with cisplatin for 48 h, the cell viability was detected as above.

**Reactive Oxygen Species (ROS)**

The LOVO and LOVO/DDP cells were seed in a 12-well plate at a density of 5 × 10^3 cells / well, and when cell proliferation reached to the Log-phase, they were treated with 1 μg/mL cisplatin and 1mM ropivacaine alone or combine, the levels of reactive oxygen species (ROS) were measured by H_2DCFDA fluorescent probe according to the manufacturer's instruction under the fluorescence microscope (Olympus IX73P2F, Japan), the quantification of average optical density was by ImageJ software.

**Mitochondrial Membrane Potential (MMP)**

The mitochondrial membrane potential (MMP) assay was based on JC-1 staining (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide). JC-1 has two forms including monomers and polymers, and the emission spectra of which are different. When the mitochondrial membrane potential reduces, the JC-1 polymer with red fluorescence decreases, and the JC-1 monomer with green fluorescence was in the cytoplasm. Cell treatment was same as ROS determination, the levels of MMP were assessed by JC-1 fluorescent probe according to the manufacturer's instruction under the fluorescence microscope (Olympus IX73P2F, Japan), the ratio of red/green fluorescence was compared after quantifying by ImageJ software.

**Wound Healing**
LOVO and LOVO/DDP cells were seeded in a 6-well plate at a density of $5 \times 10^4$ cells/well. After the density reached 80% to form a confluent monolayer, a scratch was made using 200 µl sterile pipette tip to create a wound and washed with PBS. Those above cells were then treated with 1 µg/mL cisplatin and 1mM ropivacaine alone or combinate, after incubated for 0, 48 h. The images of migrated cells were taken under an inverted microscope (Olympus IX73P2F, Japan), the width of scratch was calculated by Olympus cellSens (Olympus, Version 1.5, Japan).

Transwell Assay

The migration of LOVO and LOVO/DDP cells were detected by a boyden chambers containing 24-well Transwell plates (Corning Inc., USA) with 8 mm pore size. Those above cells were seeded at a density of $1 \times 10^4$ cells/well in the upper chambers with RPMI 1640 medium containing 5% fetal bovine serum (FBS), the bottom chamber was cultured with RPMI 1640 medium containing 10% FBS. Following incubation with 1 ug/mL cisplatin and 1mM ropivacaine alone or combinate for 48 h, cells remained on the upper side were gently removed with a cotton swab, and those migrated to the bottom surface were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The images of migrated cells were taken under an inverted microscope (Olympus IX73P2F, Japan) and the numbers of migrated cells were quantified by Image J software.

Colony Formation

LOVO and LOVO/DDP cells were cultured as describe above, quantified with a coulter particle after diluted with RPMI 1640 medium containing 10% of FBS, and aliquoted to six-well plates at a density of 300 cells/well. After treated with 1 ug/mL cisplatin and 1mM ropivacaine alone or combinate, the cells were cultured for 14 days and the culture medium was changed periodically. Finally, the cell colonies were fixed with 6% glutaraldehyde and stained with 0.1% crystal violet, the colony number and size were quantified using Image J software.

AnnexinV-FITC/PI

LOVO and LOVO/DDP cells were seeded in a 6-well plate at a density of $5 \times 10^5$ cells/well. After treated with 1 ug/mL cisplatin and 1mM ropivacaine alone or combinate, cells in each group were collected and centrifuged at 1000 rpm/min to remove the supernatant, the precipitated cells were washed by PBS for twice, and a total of 500 µL of buffer solution was added to the cell suspension. Then, 5 µL of Annexin V and 10 µL of propidium iodide were added, followed by incubation at room temperature for 30 min in dark, the apoptosis was detected by flow cytometry, and the apoptosis rate was calculated.

Western blotting

Cells were seeded into 6-well plates at a density of $5 \times 10^5$ cells/well, after treated with 1 ug/mL cisplatin and 1mM ropivacaine alone or combinate. cells in each group were collected and the protein concentration was measured with a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai,
China). A total of 40 μg proteins were subjected to protein separation by the sodium dodecyl sulfate–
polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were then transferred to a polyvinylidene
fluoride (PVDF) membrane. The membranes were incubated with 5% nonfat skim milk for 2 h. The
membranes were incubated with primary antibodies (Bax, 1:1000; Bcl-2, 1:1000; Cleaved-Caspase-3,
1:1000; Nrf-2, 1:1000; GPX4, 1:1000; SLC7A11, 1:1000) at 4 °C overnight, and then with HRP-linked
secondary antibody for 2 h at room temperature. Specific proteins were detected by enhanced
chemiluminescence assay (Bio-rad, USA), and the protein bands were quantified with Image Lab software
(Bio-rad, USA).

**Immunofluorescence (IF)**

Cells were seed in a 12-well plate at a density of 5 × 10^3 cells/well. After treated with 1 ug/mL cisplatin
and 1mM ropivacaine alone or combinate. Cells were fixed in 4% paraformaldehyde and permeabilized
with 0.2% Tween X-100. After being blocked with normal goat serum, cells were incubated with rabbit
anti-rat GPX4 (1: 200) overnight at 4 °C, and the second antibody (1: 1000) was incubated for 2 h at 37 °C
in dark. Hoechst33258 was added followed by incubation for 10 min to label nuclei. The images were
taken, and fluorescence intensity was analyzed with ImageJ software.

**FerroOrange Staining**

Cells were seeded in a 12-well plate at a density of 5 × 10^3 cells/well. After treated with 1 ug/mL cisplatin
and 1mM ropivacaine alone or combinate, for imaging of intracellular Fe^{2+}, cells were stained with serum-
free phenol red-free DMEM containing 1 µM FerroOrange for 30 min at 37 °C in a CO2 incubator.
Fluorescence measurements were conducted using fluorescence digital microscopy (Olympus IX53,
Japan). Fluorescence intensity was quantified using ImageJ.

**Molecular docking**

Structure-based virtual screening was employed with molecular docking program Autodock Vina (version
1.1.2). The PyMol (version 2.3) was used to generate the 3D schematic representation of STAT3 protein
(PDB ID: 6QHD) which was extracted by in The Research Collaboratory for Structural Bioinformatics
Protein Data Bank database. The 2D schematic representation of the interaction between ligand and
other amino acid residues was shown by LigPlus (version 2.1).

**Statistical Analysis**

Quantitative data are represented as the mean ± standard deviation (SD). Statistical analyses were
completed using SPSS program. The comparisons between the two groups were done with Student’s t-
test. The comparison among groups was performed by one- way ANOVA. A value of \( P < 0.05 \) was
considered statistically different.

**Results**
Analyze of cisplatin-sensitivity of LOVO and LOVO/DDP cells

To assess the cisplatin-sensitivity of LOVO and LOVO/DDP cells, the above cells were treated with different dose of cisplatin, the results from Fig.1A shown that cisplatin inhibit cell viability of LOVO cells in a dose-dependent manner \((P<0.01)\) and the IC50 was 1.583 \(\mu\)g/mL; but in LOVO/DDP cells, compared with the control group, the lower dose of cisplatin \((<1\ \mu\text{g/mL})\) has no effect to the cell viability \((P>0.01)\), when the concentration was larger than 2 \(\mu\text{g/mL}\), cell viability of LOVO/DDP had been decreased obviously and the IC50 was 3.281 \(\mu\text{g/mL}\), which suggested that LOVO/DDP cells had the chemotherapy-resistance to cisplatin. Subsequently, we compared the levels of ROS in LOVO and LOVO/DDP cells before and after the treatment with cisplatin. In LOVO cells, the fluorescence intensity of ROS in control group was lower, cisplatin treatment enlarged the fluorescence; however, the content of ROS in LOVO/DDP cells was not significantly increased after cisplatin treatment at the same concentration (Fig.1B), those results demonstrated that the development of cisplatin-resistance for human colorectal cancer was associated with decreased ROS reactivity to cisplatin.

Ropivacaine increased the cisplatin-sensitivity of LOVO and LOVO/DDP cells

To illustrate the anti-tumor effect of ropivacaine, above cells were treated with different dose ropivacaine for 48 h, as shown in the Fig.2A, 2B, both of LOVO and LOVO/DDP cells showed sensitivity to ropivacaine treatment, the cell viability was decreased obviously with an increase in ropivacaine concentration. Next, we detected the effect of ropivacaine in combination with cisplatin to cell viability, both in LOVO and LOVO/DDP cells, cell viability was reduced in a dose-dependent manner with the help of ropivacaine and cisplatin, especially in LOVO/DDP cells, the cell viability was significant decline from 94.6% to 68.7% (Fig.2C).

Ropivacaine inhibited the proliferation and migration of LOVO and LOVO/DDP cells combined with cisplatin

The reduction of cisplatin-sensitivity is characterized by increasing the proliferation and migration of tumors, so we detected the effect of ropivacaine combined with cisplatin to cell proliferation and migration. Wound Healing and Transwell assay were used to assess the migration, as shown in Fig.1A, compared to the control group, cisplatin inhibited the healing of LOVO cells towards the middle, and the width of scratch was obviously increased after the treatment of ropivacaine combined with cisplatin. However, the wound healing was not inhibited in LOVO/DDP cells after cisplatin alone treatment, only when cisplatin and ropivacaine act in combination, the migration of LOVO/DDP has been decreased significant. The results of Transwell assay were the same as wound healing, numbers of migrate cells in LOVO and LOVO/DDP cells were significant reduced after the treatment of cisplatin combined with ropivacaine (Fig.3B). The assessment of cell proliferation was by colony formation, Results from Fig.3C demonstrated that cisplatin inhibited cell proliferation of LOVO cells and had no effect on LOVO/DDP cells, it showed obvious inhibitory effect on LOVO and LOVO/DDP cell proliferation with the combination of cisplatin and ropivacaine. Additionally, ropivacaine combined with cisplatin down-regulated MMP-9 expression in LOVO and LOVO/DDP cells compared to cisplatin alone treatment \((P<0.01)\) ((Fig.3C, 3D).
Thus, the results showed that ropivacaine inhibited the proliferation and migration of LOVO and LOVO/DDP cells combined with cisplatin.

**Ropivacaine promoted the synthesis of ROS and mitochondrial dysfunction in combination with cisplatin**

To illustrate the effect of ropivacaine combined with cisplatin on the synthesis of ROS and mitochondrial function, the fluorescent probes of ROS and mitochondrial membrane potential (MMP) were detected, results from Fig. 4A showed that ropivacaine combined with cisplatin increased the green fluorescence intensity of ROS in LOVO and LOVO/DDP cells compared to cisplatin alone treatment ($p<0.01$), which demonstrated that ropivacaine and cisplatin acted in combination promoted the synthesis of ROS. The damage mitochondria is characterized by the reduction of MMP, which will decrease the JC-1 polymer in cytoplasm. As shown in Fig. 4B, ropivacaine combined with cisplatin decreased the red fluorescence of JC-1 polymer in LOVO and LOVO/DDP cells compared to cisplatin alone treatment ($p<0.01$), which demonstrated that ropivacaine and cisplatin acted in combination promoted mitochondrial dysfunction.

**Ropivacaine promote apoptosis of LOVO and LOVO/DDP cells in combination of cisplatin**

To evaluate the apoptotic effect of ropivacaine combined with cisplatin, AnnexinV-FITC/PI flow cytometry was used to measure the levels of apoptosis. As shown in Fig. 5, the results displayed that cisplatin increased the apoptosis rate in LOVO cells, and in LOVO/DDP cells, cisplatin slight increased apoptosis compared to the control group, but the difference was not statistically significant ($p>0.05$). However, ropivacaine combined with cisplatin had a significantly higher apoptosis ration than cisplatin alone treatment in LOVO and LOVO/DDP cells ($p<0.01$). Those results demonstrated that ropivacaine promoted apoptosis of LOVO and LOVO/DDP cells in combination of cisplatin.

**Ropivacaine promote ferroptosis of LOVO and LOVO/DDP cells in combination of cisplatin**

Ferroptosis is characterized by lipid peroxidation and mitochondrial damage, the above results demonstrated that ropivacaine combined with cisplatin increased the synthesis of ROS and induced mitochondrial dysfunction, so we would like to detect the effect of ropivacaine combined with cisplatin on ferroptosis, the results from Fig. 6A, 6B showed that compared to cisplatin group, ropivacaine combined with cisplatin promoted the ferroptosis of LOVO and LOVO/DDP cells by down-regulating the anti-ferroptosis proteins including Nrf-2, GPX4 and SLC7A11. Except for western blot, the levels of GPX4 in LOVO and LOVO/DDP cells were also detected by immunofluorescence, the results from Fig. 6C, 6D were consistent as above, ropivacaine combined with cisplatin inhibited the expression of GPX4 protein to promote ferroptosis of LOVO and LOVO/DDP cells than cisplatin alone treatment. Finally, a fluorescent probe of Fe$^{2+}$ was used after the treatment with ropivacaine or cisplatin, ropivacaine combined with cisplatin increased the levels of Fe$^{2+}$ in LOVO and LOVO/DDP cells than cisplatin alone treatment (Supplement.Fig.1). Those results demonstrated that ropivacaine promote ferroptosis of LOVO and LOVO/DDP cells in combination of cisplatin.

**Ropivacaine combined with cisplatin inhibit SIRT1 protein expression**
To illustrate the effect of ropivacaine on SIRT1 expression, the levels of SIRT1 protein were evaluated using Western-blot. As shown in Fig. 7A, the expression of SIRT1 was downregulated by cisplatin in LOVO cells, and ropivacaine combined with cisplatin obviously inhibited SIRT1 protein compared to cisplatin alone treatment ($p<0.01$). In LOVO/DDP cells, cisplatin alone treatment has no effect of SIRT1 protein. However, ropivacaine combined with cisplatin significantly downregulated the expression of SIRT1, which suggested that ropivacaine improve the anti-tumor effect of cisplatin via the inhibition of SIRT1. Subsequently, the autodock-Vina was to examine the interaction between SIRT1 and ropivacaine. As shown in Fig.7E-7G, ropivacaine could bind to SIRT1, the affinity between them was -8.56 and the combined pocket was composed of Ile411, Phe297, His363, Ile347, Gln345, Ala262, Arg274, Asn346, Phe273, Val445, Phe414 and Val412. They interacted through hydrophobic bonds to inhibit the expression of SIRT1.

**Discussion**

Numerous studies have shown that the perioperative use of local anesthetics can be benefit to cancer patients and have been demonstrated to reduce the risk of metastasis and recurrence for various cancers in vivo or vitro experiments [22-24]. In particular, the amide-linked local anesthetics have been shown to directly inhibit tumor growth, proliferation, and invasion. Bupivacaine exerted an anti-tumor role to suppress the progression of gastric cancer through regulating circ_0000376 /miR-145-5p axis [25]. And lidocaine suppresses the growth of cancer cells through increasing GDF-15 and TRIB3 expression [26]. Besides, ropivacaine has been found to have inhibitory activity and sensitizing effects when used in combination with conventional chemotherapeutics toward cancer cells, but the specific mechanism remains unclear [27]. Ropivacaine inhibited the growth and progression of various tumors through multiple pathways, a study conducted by Baptista-Hon et al. reported that ropivacaine blocked the NaV1.5 VGSC in colon cancer cells to inhibit the invasion [28], and it also has significant antitumor effects on primary (SW480) and metastatic (SW620) colon cancer cells [29], those studies suggested that ropivacaine has a potential application in controlling colon cancer growth.

Our results found that ropivacaine inhibit the growth of cisplatin-sensitive colorectal cancer (CRC) cell line LOVO cells and cisplatin-resistant LOVO/DDP cells in a dose-dependent manner. And ropivacaine combined with cisplatin obviously decreased the cell viability of LOVO and LOVO/DDP cells when compared to cisplatin alone treatment. Subsequently, we also demonstrated ropivacaine combined with cisplatin inhibit the proliferation and migration of LOVO and LOVO/DDP cells, it' obvious that the inhibitory effect of the combination is increased compared with cisplatin alone. Yang et al. reported that ropivacaine inhibited angiogenesis of human lung tumor-associated endothelial cells by disturbing the complex II located in the mitochondrial respiration chain, inducing the damaged mitochondria and excessive production of ROS [30]. In our study, we found that the production of ROS existed a clear difference in LOVO and LOVO/DDP cells after cisplatin alone treatment, but ropivacaine combined with cisplatin induced the decrease of mitochondrial membrane potential and the overproduce of ROS, which finally leaded to the mitochondrial damage. Another study from Gong et al. reported that ropivacaine inhibited the survival and colony formation of breast cancer cells through inducing mitochondrial...
dysfunction and oxidative stress [13]. All in all, the induction of damaged mitochondria and oxidative stress in cancer cells is an effective strategy to combat the drug resistance.

Cisplatin has been shown to impair DNA repair by crosslinking with purine bases on DNA, leading to the cell death of cancer cells, it is widely used in clinical practice as the first-line agent for the chemotherapy of colon cancer [31]. However, the drug resistance of cisplatin limited its clinical application [32]. At present, the molecular mechanism underlying cisplatin resistance are complex, including the inactivation of apoptosis, the reduction in the intracellular accumulation of the platinum, and the activation of epithelial-mesenchymal transition [4]. Many studies have shown that the cisplatin-resistant cancer cells have the lower levels of cisplatin-induced apoptosis than cisplatin-sensitive cells [33-35]. Liu et al. found that berberine in combination with cisplatin induced DNA damage and apoptosis in ovarian cancer cells [34], another study from Guo et al. reported HOTAIR promoted cisplatin resistance of osteosarcoma cells by regulating apoptosis via mir-106a-5p/stat3 axis [36]. Our results were consistent with above, cisplatin significantly increased the apoptotic levels of LOVO cells, but in LOVO/DDP cells, this effect was not obvious. However, ropivacaine combined with cisplatin significantly upregulated the apoptotic levels of LOVO and LOVO/DDP cells. Which suggested that ropivacaine enhanced the cisplatin sensitivity of colon cancer cells by the activation of apoptosis. In a word, prompting apoptosis of cancer cells is an effective measure to overcome cisplatin resistance for tumors.

In addition, through detecting the mitochondrial member potential and the levels of ROS in LOVO and LOVO/DDP cells before and after the treatment with cisplatin and ropivacaine, we identified that ropivacaine could enhance the antitumor effects of cisplatin by promoting mitochondrial damage and ROS overproduce. As a new model of cell death, ferroptosis can elevate ROS and MDA levels, and finally results in overwhelming lipid peroxidation to cause cell death [37, 38]. Cisplatin have been confirmed to be an inducer for both ferroptosis and apoptosis in A549 and HCT116 cells [39]. Therefore, the induction of ferroptosis is considered as a promising strategy for eradicating aggressive malignancies resistant to traditional chemotherapy. Fu et al. found that activation transcription factor 3 (ATF3) may sensitize gastric cancer cells to cisplatin by induction of ferroptosis via blocking Nrf2/Keap1/xCT signaling [40]. And Du et al. also confirmed that the combination of dihydroartemisinin (DHA) and cisplatin could intensively strengthen the cytotoxicity of cisplatin to inhibit the growth of pancreatic ductal adenocarcinoma cells via inducing ferroptosis [41]. In addition, we detected the expression of ferroptosis-related proteins, and demonstrated that ropivacaine combined with cisplatin increased the ferroptosis levels of LOVO and LOVO/DDP cells via downregulating Nrf-2, GPX4 and SLC7A11 proteins when compared to cisplatin alone treatment, we also found the accumulation of Fe2+ was elevated in combination with ropivacaine and cisplatin. Furthermore, a study conducted by Lu et al. reported that ropivacaine could directly induce ferroptosis in ovarian cancer cells to restrain the cell stemness [42]. Those results suggested that ropivacaine enhanced cisplatin sensitivity to colorectal cancer cells via prompting ferroptosis.

As we know, the mitochondrial damage is the key link in apoptosis and ferroptosis, the silencing information regulator 2 related enzyme 1 (SIRT1), as an NAD+-dependent deacetylase, has been
demonstrated to protect the damaged mitochondria via the inhibition of oxidative stress [43]. However, through the small molecule docking, we found that ropivacaine could target binding to SIRT1 and decreased its expression, which led to ROS overproduce and mitochondrial member potential decreased. Many studies also confirmed that the activation of SIRT1 could alleviate apoptosis or ferroptosis. For instance, quercetin could attenuate the apoptosis of rat chondrocytes via upregulating the proteins expression of SIRT1 [44], and irisin protect the acute kidney injury by inhibiting ferroptosis, it mainly reduced the ROS production, iron content and MDA levels via activating the SIRT1/Nrf-2 signaling pathway [45]. In a word, the induction of apoptosis and ferroptosis from ropivacaine is thought to be closely related to silencing SIRT1.

In summary, our study was the first to detect the effect of ropivacaine on cisplatin-resistant colon cancer cells, we found that ropivacaine combined with cisplatin decreased the cell viability, mitochondrial member potential and promoted ROS production, apoptosis and ferroptosis in LOVO and LOVO/DDP cells, ropivacaine enhanced the cisplatin sensitivity to CRC cells compared with cisplatin alone treatment, and finally we put forward a hypothesis that ropivacaine increase the cytotoxicity of cisplatin is by silencing SIRT1, which may require further study. In a word, this study might provide a new treatment strategy for cisplatin resistance in CRC. But there are many limitations, we need to add agonists and inhibitors of apoptosis or ferroptosis to clarify the role of ropivacaine and we also need to further explore the sensitizing effect of ropivacaine to cisplatin in vivo experiment, which will be our follow-up research direction.

**Declarations**

**Funding statement**

This study was supported by the National Natural Science Foundation of China (81703015), Hubei Provincial Natural Science Foundation of China (2021CFB582), Health Commission of Hubei Province scientific research project (WJ2021M069), Educational Commission of Hubei Province scientific research project (D20212103), The Foundation of Health Commission of Hubei Province (WJ2023F077, WJ2023F079)

The Science and Technology Development Project of Xiangyang (2021YL23, 2021ZD12), and Innovative Research Program of Xiangyang No.1 People's Hospital (XYY2021M01, XYY2021M12).

**Disclosure statement**

The authors declare no conflicts of interest regarding this study and publication.

**Authors contributions**

LHY and HPC designed the studies, ZWB and ZZ undertook the cell experiments, ZL and ZQ undertook the molecular biology testing, ZQ and WQS undertook the molecular docking. ZWB, ZZ, DXD analyzed data and wrote the draft of manuscript. ZL, ZY and XQQ undertook the revision of manuscript.
Availability of data and materials

Data that support the study findings are available from the corresponding author upon reasonable request.

References


Figures

Figure 1

Comparing the differences in cisplatin-sensitive and cisplatin-resistant colon cancer cells. A. LOVO cells and LOVO/DDP cells were before and after treated with cisplatin, the cell viability was detected by CCK-8. B. The intracellular ROS was detected by H$_2$DCFDA fluorescent probe after the treatment with cisplatin. The results are presented as the mean ± SD (n = 3), where * $p < 0.05$, ** $p < 0.01$, vs control group.
Figure 2

The detection of cell viability after the treatment with ropivacaine and cisplatin in alone or in combination. A, B. LOVO cells and LOVO/DDP cells were treated with ropivacaine for 24 h, the cell viability was detected by CCK-8. C LOVO cells and LOVO/DDP cells were treated with ropivacaine and cisplatin in alone or in combination, the cell viability was detected by CCK-8. The results are presented as the mean ± SD (n = 3), where * \( p < 0.05 \), ** \( p < 0.01 \), vs control group. ## \( p < 0.01 \) vs Cisplatin group.
**Figure 3**

**Ropivacaine combined with cisplatin inhibited the proliferation and migration of LOVO cells and LOVO/DDP cells.** LOVO cells and LOVO/DDP cells were before and after treated with cisplatin and ropivacaine, the proliferative capacity was assessed by Wound Healing (A) and Colony Formation (C), the migration was detected with Transwell Assay (B), Western-blot was used to detect MMP-9 protein expression (D, E). The results are presented as the mean ± SD (n = 3), where * $p < 0.05$, ** $p < 0.01$, vs control group. ## $p < 0.01$ vs Cisplatin group.
Figure 4

Ropivacaine prompted cisplatin-induced oxidative stress and mitochondrial dysfunction of LOVO cells and LOVO/DDP cells. A. LOVO cells and LOVO/DDP cells were before and after treated with cisplatin and ropivacaine, the intracellular ROS was detected by H$_2$DCFDA fluorescent probe. B. LOVO cells and LOVO/DDP cells were before and after treated with cisplatin and ropivacaine, the mitochondrial membrane potential (MMP) was detected by a JC-1 detection kit. C. The ratio of average optical density
Figure 5

**Ropivacaine prompted cisplatin-induced apoptosis of LOVO cells and LOVO/DDP cells.** A. LOVO cells and LOVO/DDP cells were before and after treated with cisplatin and ropivacaine, the levels of apoptosis were detected by flow cytometry. B. The ratio of the numbers of apoptotic cells than total cells. The results are presented as the mean ± SD (n = 3), where * \( p < 0.05 \), ** \( p < 0.01 \), vs control group. ##\( p<0.01 \) vs Cisplatin group.
Figure 6

**Ropivacaine prompted cisplatin-induced ferroptosis of LOVO cells and LOVO/DDP cells.** A. LOVO cells and LOVO/DDP cells were before and after treated with cisplatin and ropivacaine, the expression of Nrf-2, GPX4, SLC7A11 proteins were detected by Western-blot. B. Quantification of the gray values in graph A. C. The expression of GPX4 was detected by immunofluorescence. D. The ratio of the average optical density...
in graph C. The results are presented as the mean ± SD (n = 3), where * $p < 0.05$, ** $p < 0.01$, vs control group. ## $p < 0.01$ vs Cisplatin group.

### Figure 7

**Ropivacaine combined with cisplatin inhibited SINT1 protein expression.** A, B. LOVO cells and LOVO/DDP cells were before and after treated with cisplatin and ropivacaine, the expression of SIRT1 protein were detected by western-blot, C, D. Quantification of the gray values in graph A, B. The computational modeling of ropivacaine binding to the SIRT1, and the affinity between them was -8.56, E. 3D structure display of ropivacaine bonded to SIRT1, F. local enlarged image of graph E, G. the 2D structure model of ropivacaine bonded to SIRT1. The results are presented as the mean ± SD (n = 3), where * $p < 0.05$, ** $p < 0.01$, vs control group. ## $p < 0.01$ vs Cisplatin group.
Figure 8

The schematic diagram illustrating ropivacaine facilitate cisplatin-induced apoptosis and ferroptosis.
Ropivacaine enhanced cisplatin-induced the overproduction of ROS and dysfunctional mitochondria via the inhibition of SIRT1 in LOVO/DDP cells, subsequently, ROS and dysfunctional mitochondria caused apoptosis and ferroptosis, so that ropivacaine enhanced the cisplatin sensitivity of colon cancer via ROS-mediated apoptosis and ferroptosis, which may be achieved by suppressing SIRT1.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplement1.tiff