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Low-energy shock waves promote the cisplatin chemosensitivity of human osteosarcoma MNNG/HOS cells via the P2X7/Akt/mTOR pathway

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Background: The current dilemma of osteosarcoma treatment is the resistance of chemotherapeutic drugs after long-term usage, which also introduces life-threatening side effects.

Methods and results: To minimize chemoresistance in osteosarcoma patients, the authors applied shock waves (SWs) to human osteosarcoma MNNG/HOS cells, then evaluated the cell viability and extracellular ATP levels, and further investigated the effect of SWs on cisplatin (DDP) cytotoxicity in MNNG/HOS cells. The authors' results showed that 400 SW pulses at 0.21 mJ/mm^2 exhibited little influence on the MNNG/HOS cell viability. In addition, this SW condition significantly promoted the extracellular ATP release in MNNG/HOS cells. Importantly, low-energy SWs obviously increased Akt and mammalian target of rapamycin (mTOR) phosphorylation and activation in MNNG/HOS cells, which could be partially reversed in the presence of P2X7 siRNA. The authors also found that low-energy SWs strongly increased the DDP sensitivity of MNNG/HOS cells in the absence of P2X7.

Conclusions: For the first time, the authors found that SW therapy reduced the DDP resistance of MNNG/HOS osteosarcoma cells when the ATP receptor P2X7 was downregulated. SW therapy may provide a novel treatment strategy for chemoresistant human osteosarcoma.

Keywords: DDP, human osteosarcoma, P2X7, shock wave

Introduction

Osteosarcoma is the most common type of bone cancer, accounting for more than 2% of all paediatric tumour cases[1]. Due to chemoresistance after long-term exposure to chemotherapeutic drugs, the 5-year survival rate of osteosarcoma patients is less than 70%[2,3]. Meanwhile, obvious life-threatening side effects of high doses of chemotherapeutic drugs are observed as well. Therefore, the discovery of a novel method to replace conventional chemotherapeutic drugs is urgently needed.

To overcome the chemoresistance of osteosarcoma, several novel strategies have been developed, including shock wave (SW) therapy[4]. SWs introduce a great positive pressure, further increase the mammalian cell membrane permeability, and transiently induce membrane pore formation, thus allowing macromolecular drugs to enter cells and elicit their therapeutic effects[5].

HIGHLIGHTS

- Low-energy shock waves (SWs) significantly elevate the extracellular ATP concentration in human osteosarcoma MNNG/HOS cells.
- Low-energy SWs notably increase the activation of Akt and mammalian target of rapamycin (mTOR) in MNNG/HOS cells, which is partially dependent on P2X7.
- Low-energy SWs sensitize MNNG/HOS cells to cisplatin (DDP) in the absence of P2X7.

Currently, SWs are known to enhance the cytotoxicity of chemotherapeutic drugs in several types of carcinoma, including breast[6], bladder[7], and prostate cancers[8], although SWs alone show little inhibitory influence on tumour growth. In a Mat B-III syngeneic rat breast cancer model, SWs and the chemical protoporphyrin IX have been demonstrated to decrease the tumour size with stronger necrotic and apoptotic clinical histological features compared to protoporphyrin IX alone[6]. In addition, low-energy SWs have been shown to promote intravesical epirubicin delivery in bladder cancer, followed by reduced metastasis and dysplasia formation[7]. Moreover, SW treatment has been reported to inhibit the activation of cancer-associated fibroblasts as well as to decrease androgen-resistant prostate cancer cell growth and invasion[8].

Our previous studies have shown that SWs promote doxorubicin and methotrexate (MTX) uptake by U2OS cells through P2X7 receptor-mediated cell membrane channel opening[9]. P2X7 is the extracellular ATP receptor universally expressed on the cell surface of various mammalian cells[10]; this receptor is abnormally elevated in breast cancer and is involved in breast cancer growth and metastasis through activating multiple

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downstream cellular signalling pathways[11]. P2X7 is activated once it binds to extracellular ATP to promote the survival and proliferation of multiple cancer cell lines via the ERK1/2 and JNK pathways, including in glioma[12], pancreatic cancer[13], and ovarian cancer[14]. Although SWs have been demonstrated to obviously enhance the cytotoxicity of chemotherapeutic drugs, including doxorubicin and MTX in osteosarcoma U2OS cells through P2X7[9], the underlying mechanism of action of SWs in chemoresistant osteosarcoma cells has not been fully elucidated.

In this study, we investigated the effect of low-energy SWs on the vitality of osteosarcoma MNNG/HOS cells. In addition, we determined whether low-energy SWs promoted MNNG/HOS cell proliferation through extracellular ATP release and the Akt/mammalian target of rapamycin (mTOR) pathway and whether this effect was dependent on the ATP receptor P2X7. Furthermore, we analyzed the effect of cisplatin (DDP) in the presence of low-energy SWs on MNNG/HOS cell apoptosis.

Materials and methods

Cell culture

The human osteosarcoma cell line MNNG/HOS was purchased from the Cell Bank of the Shanghai Institute of Cell Biology (Chinese Academy of Sciences) and maintained in minimal essential medium (HyClone) containing 10% foetal bovine serum (BI), 1.5 g/l $NaHCO_3$, 0.11 g/l sodium pyruvate, and 1% penicillin–streptomycin (HyClone).

RNA interference

MNNG/HOS cells were seeded into 6-well cell culture plates and transfected with siRNA targeting P2X7 using jetPRIME (Polyplus). Cells were harvested for western blot analysis 48 h later. The following siRNA oligonucleotides were synthesized and obtained from GenePharma Inc. (Shanghai, China): P2X7 sense, 5′-CCGAGAAACAGGCGAUAAUTT-3′ and anti-sense, 5′- AUUAUCGCCUGUUUCUCGGTT-3′.

Cell counting kit-8 (CCK-8) cell viability assay

The cell viability was evaluated by using a CCK-8 (New Cell & Molecular Biotech). Briefly, 3000 MNNG/HOS cells per well were transferred to a 96-well cell culture plate and then exposed to 400 SW pulses (HK.SWT-300) at levels of 0, 0.11, 0.15, 0.21, and 0.23 mJ/mm^2. After the 30-min treatment, the CCK-8 reagent was added to the cells, the mixture was incubated at 37°C for 60 min, and the absorbance at 450 nm was measured. The cell viability is presented as the percentage of optical density (OD) value of SW treatment divided by the OD value in untreated cells.

Annexin V–propidium iodide (PI) cell apoptosis assay

A cell apoptosis assay was performed using an annexin V-FITC/PI apoptosis detection kit (Vazyme Biotech, Nanjing, China). Briefly, 5×10^4 MNNG/HOS cells carrying P2X7 siRNA were treated with 10 μM DDP and then exposed to 400 SW pulses at 0.21 mJ/mm^2. Next, the cells were suspended and washed with phosphate-buffered saline, stained with Annexin V-FITC reagent, and subjected to flow cytometric analysis (BD, FACSCalibur).

Western blot

The whole-cell lysates were harvested using radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. After measuring the protein concentration using a bicinchoninic acid assay, the cellular proteins were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis. After electrotransfer to polyvinylidene difluoride membranes, the membranes containing cellular proteins were incubated with primary antibodies against the target proteins. The primary antibodies used in this study were as follows: p-Akt (Cell Signalling Technology), p-mTOR (Cell Signalling Technology), P2X7 (Bioss), and β-actin (Cell Signalling Technology). After reacting with horseradish peroxidase-conjugated anti-rabbit IgG, the proteins of interest were visualized with enhanced chemiluminescence reagents and normalized using ImageJ software.

RNA extraction and reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen), according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using an RT-PCR kit (Takara, Beijing, China) and then subjected to real-time PCR analysis using a Q6 sequence detector with SYBR green qPCR mix. The following primer sequences were designed using primer Express 4 (Life Technologies) and used in this study: AKT forward, 5′-ATCGCTTCTTTGCCGGTAT-3′; AKT reverse, 5′-TCTTGGTCAGGTGGTGTGAT-3′; mTOR forward, 5′-GCGACACCGAATCAATCAT-3′; mTOR reverse, 5′-TTTCTTCATGGGTCCTGTTT-3′; GAPDH forward, 5′-GGAGCGAGATCCCTCCAAAAT-3′; and GAPDH reverse, 5′-GGCTGTTGTCATACTTCTCATGG-3′. The results are shown as the mean of three independent experiments.

ATP measurement

The cellular ATP level was measured using an ATP enzyme-linked immunosorbent assay kit (Shanghai Heng Yuan Biological Technology). Briefly, 1×10^6 MNNG/HOS cells were exposed to SWs with and without $GdCl_3$ or Brefeldin A to block ATP release, then incubated with cell Titer-LumiTM Plus reagent for 10 min, and finally the OD value at 450 nm was measured by a microplate reader.

Statistical analysis

All data were analyzed utilizing SPSS17.0 software and presented as the mean ± standard deviation (SD). Comparison between two groups was determined by the Student's *t* test, and comparison between more than two groups was assessed by one-way analysis of variance. A *P* value less than 0.05 was regarded as statistically significant.

Results

Effect of SWs on MNNG/HOS cell viability

To optimize the SW conditions of human osteosarcoma cells, MNNG/HOS cells were treated with 400 SW pulses at levels of 0, 0.11, 0.15, 0.21, and 0.23 mJ/mm^2, and then the cell viability was determined by a CCK-8 assay. A cell viability of more than 95%

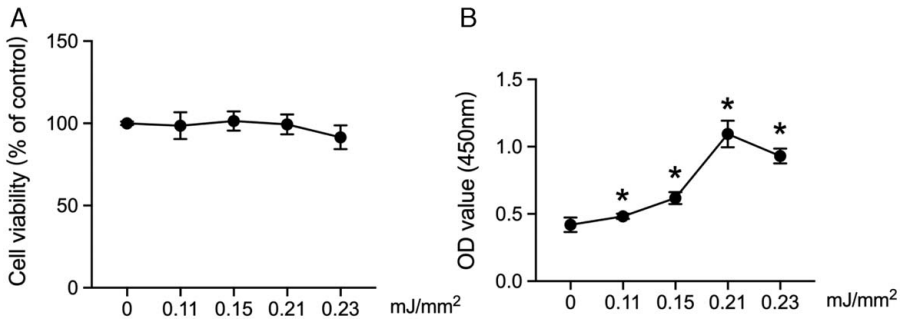

Figure 1. Optimization of SW conditions for human osteosarcoma MNNG/HOS cells. MNNG/HOS cells were exposed to 400 SW pulses at different levels for 0 h (A) and 24 h (B). The cell viability was evaluated using a cell counting kit-8 kit. Data are shown as the mean ± SD, $n = 5$, $^*P < 0.05$, one-way analysis of variance. OD, optical density. SW, shock wave.

was detected after treatment with 400 SW pulses at levels of 0, 0.11, 0.15, and 0.21 mJ/mm^2, which was considered as no effect on the cell viability (Fig. 1A). Therefore, the condition of 400 SW pulses at 0.21 mJ/mm^2 was selected for further analysis in our study. Furthermore, the cell proliferation was increased gradually after SW pulses at levels of 0.11 mJ/mm^2 to 0.21 mJ/mm^2 and decreased obviously at a level of 0.23 mJ/mm^2 compared to a level of 0.21 mJ/mm^2 (Fig. 1B), suggesting that low-energy SW pulses could promote MNNG/HOS cell proliferation.

Low-energy SWs enhance extracellular ATP release

Our previous study showed that SW treatment promoted extracellular ATP release from osteosarcoma U2OS cells[9]. Therefore, to investigate if the extracellular ATP concentration in MNNG/HOS cells was also increased after SW exposure, 1×10^6 MNNG/HOS cells were treated with 400 SW pulses at 0.21 mJ/mm^2.

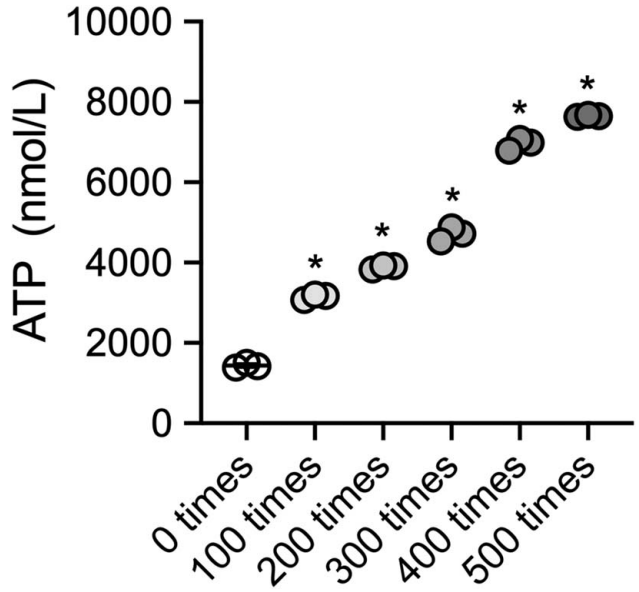

Figure 2. Low-energy SWs induced extracellular ATP release in MNNG/HOS cells. Cells were treated with SWs, as indicated, and the ATP level was determined by an enzyme-linked immunosorbent assay kit for ATP. Data are shown as the mean ± SD, $n = 3$, $^*P < 0.05$, one-way analysis of variance. SWs, shock waves.

We observed that MNNG/HOS cells treated with SWs had significantly elevated extracellular ATP levels and increased in a dose-dependent manner (Fig. 2), which was consistent with our previous findings in U2OS cells.

Low-energy SWs activate the Akt/mTOR pathway, which is dependent on P2X7

Our previous study showed that the increasing level of extracellular ATP was partially responsible for the effect of SWs on the viability of U2OS cells. Therefore, to examine whether extracellular ATP is also involved in the effect of SWs on MNNG/HOS cells, we established a P2X7-deficient MNNG/HOS cell line using P2X7 RNA interference. The P2X7 knockdown in MNNG/HOS cells was confirmed by the fact that there was a significantly lower P2X7 protein level in P2X7 siRNA-treated cells compared to control siRNA-treated cells (Fig. 3A).

To further explore the potential mechanism of the effect of SWs on MNNG/HOS cells, MNNG/HOS cells were exposed to 400 SW pulses at 0.21 mJ/mm^2 in the presence and absence of P2X7. The western blot results showed that the p-Akt and p-mTOR levels were significantly decreased in P2X7 siRNA-treated MNNG/HOS cells compared to untreated cells, but the levels were notably increased in MNNG/HOS cells exposed to SWs compared to untreated cells (Fig. 3B). The levels of p-Akt and p-mTOR were partially reversed in P2X7 siRNA- and SW-double-treated cells compared to P2X7 siRNA-alone-treated MNNG/HOS cells (Fig. 3B).

To further confirm our findings, the mRNA expression levels of Akt and mTOR were examined. Consistent with the western blot results, the mRNA expression levels of Akt and mTOR were significantly reduced in P2X7 siRNA-treated cells, but they were obviously elevated in SW-exposed cells compared to untreated cells (Fig. 3C, D). However, only the Akt level was reversed in P2X7 siRNA- and SW-dual-treated cells compared to P2X7 siRNA-transfected cells (Fig. 3C, D).

Low-energy SWs promote DDP-induced MNNG/HOS cell apoptosis

Our previous study showed that SWs enhanced MTX cytotoxicity in U2OS cells; therefore, we investigated the effect of SWs on other chemotherapeutic drugs. DDP is a chemotherapeutic drug for osteosarcoma that is widely used in the clinic. MNNG/HOS cells were incubated with DDP followed by 400 SW pulses at

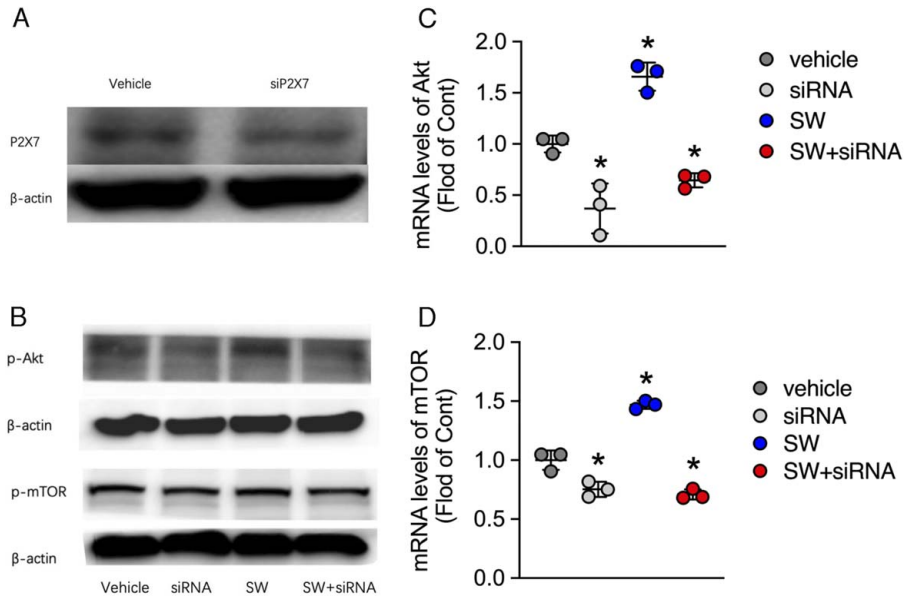

Figure 3. Low-energy SWs activated the Akt/mammalian target of rapamycin (mTOR) pathway through P2X7. (A) A western blot assay confirmed that P2X7 was knocked down after cells were transfected with P2X7 siRNA. (B) The p-Akt and p-mTOR levels after treatment with low-energy SWs and P2X7 siRNA were assessed by a western blot assay. (C) The mRNA expression levels of Akt and mTOR after treatment with low-energy SWs and P2X7 siRNA were assessed by a quantitative polymerase chain reaction assay. Data are shown as the mean ± SD, $n = 3$, $^*P < 0.05$, one-way analysis of variance. SWs, shock waves.

0.21 mJ/mm^2 in the presence and absence of P2X7 siRNA, and it was observed that DDP significantly induced MNNG/HOS cell apoptosis compared to untreated cells. In addition, SWs further increased cell apoptosis in the presence of DDP, and the highest cell apoptosis was observed with the combined treatment of SWs, DDP, and P2X7 siRNA (Fig. 4), suggesting that P2X7 mediates the cell apoptosis induced by SWs and DDP.

Discussion

In this study, we showed that low-energy SWs did not induce significant osteosarcoma cell MNNG/HOS death, as more than 95% of the total cell viability was observed when MNNG/HOS cells were treated with 400 SW pulses at 0.21 mJ/mm^2. As a result, we applied 400 SW pulses at 0.21 mJ/mm^2 for further analysis. Low-energy SWs increased MNNG/HOS cell proliferation as well as elevated extracellular ATP release in a dose-dependent manner. Because our previous data showed that SW-promoted ATP release was mediated by P2X7, we transfected P2X7 siRNA into MNNG/HOS cells to investigate the mechanism of action of SWs. After confirming that the P2X7 level was decreased in MNNG/HOS cells, we found that the p-Akt and p-mTOR levels were significantly decreased by P2X7 siRNA but greatly increased by low-energy SWs. More importantly, the lower levels of p-Akt and p-mTOR by P2X7 siRNA were partially reversed when MNNG/HOS cells were treated with P2X7 siRNA in the presence of low-energy SWs, which was supported by the fact that the Akt mRNA expression level also showed a similar changing trend as the p-Akt and p-mTOR protein levels when exposed to P2X7 siRNA, low-energy SWs alone, and P2X7 siRNA in the presence of low-energy SWs. In addition, SWs significantly promoted the proapoptotic effects of DDP on MNNG/HOS cells in the presence of P2X7 siRNA.

SW therapy has been widely used in the clinical treatment of various cancers, including breast, bladder, and prostate cancers[6–8]. Our previous study showed that SWs promoted extracellular ATP release in U2OS osteosarcoma cells and further sensitized U2OS cells to MTX cytotoxicity[9]. In the current investigation, we also observed a similar synergetic effect of SWs and DDP on MNNG/HOS osteosarcoma cells in the absence of P2X7, suggesting that the sensitizing effect of SWs to chemotherapy in osteosarcoma cells depended on P2X7 by a universal mechanism of action for SWs. P2X7, an extracellular ATP receptor, has been reported to enhance the growth and metastasis of MNNG/HOS osteosarcoma cells and to be mediated by Akt and mTOR cell signalling[15]. Although SWs alone did not show a significant inhibitory effect on the tumour growth of breast carcinoma, animal model studies have shown that SWs greatly reduce chemoresistant cancer cell proliferation and metastasis, with a significantly lower usage of chemotherapeutic drugs[6]. The molecules involved in cancer cell invasion, including hypoxia-inducible factor 1-alpha and vascular endothelial growth factor, which are also the downstream executors of mTOR, have been demonstrated to be significantly decreased in SW-treated mice compared to untreated animals[16].

A high expression level of P2X7 is detected in most cancer cells and is responsible for cancer cell invasion and distant metastasis[10]. Several crucial cell signalling mediators of P2X7, including Akt and mTOR, play key roles in tumour cell metastasis[15,17]. Our results confirmed that Akt and mTOR were the downstream targets of P2X7 in low-energy-SW-treated MNNG/HOS osteosarcoma cells, as shown in Fig. 2. P2X7 not only affected the phosphorylation and activation of Akt and mTOR but regulated the transcription of these two genes as well. P2X7 has been shown to contribute to cytotoxic effects on human melanoma and mesothelioma cells through PI3K/Akt signalling under high ATP levels[18,19], but our results showed that P2X7 inhibited PI3K/Akt pathway activity even if low-energy SWs

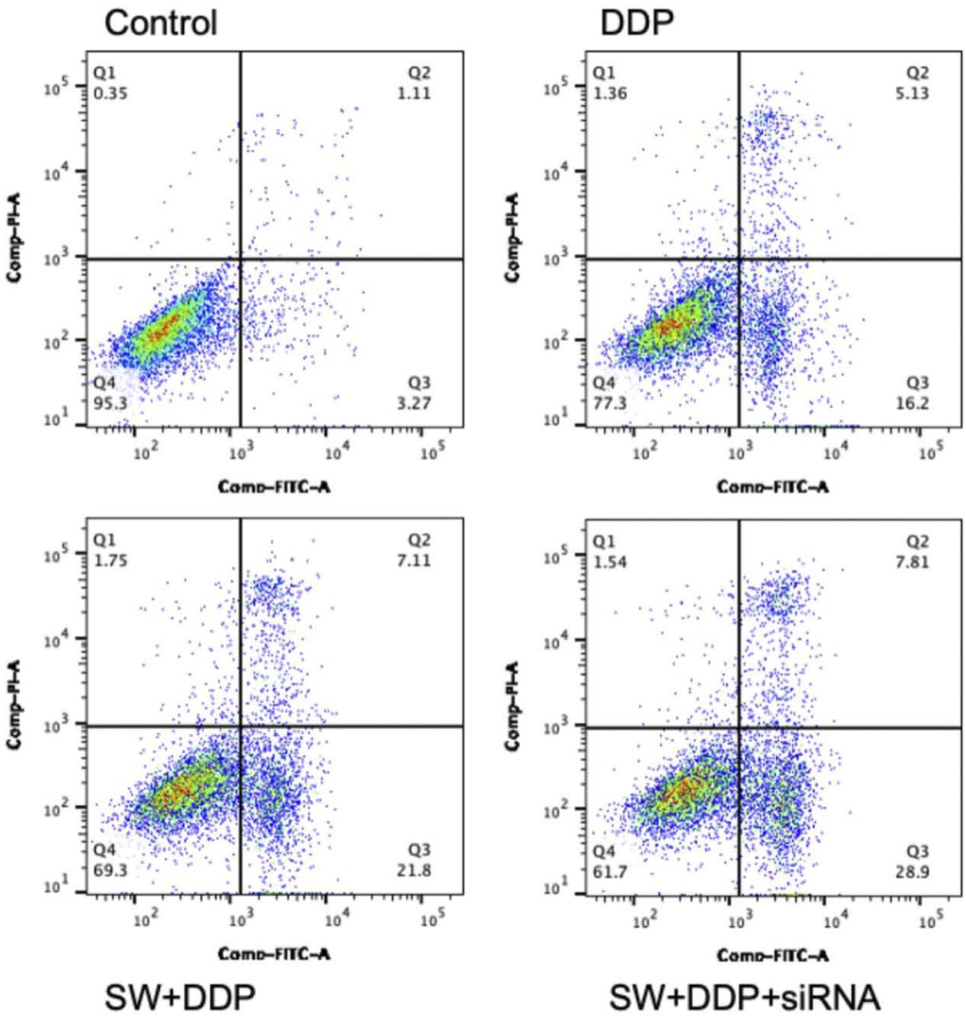

Figure 4. Low-energy shock waves (SWs) enhanced cisplatin (DDP)-mediated MNNG/HOS cell apoptosis. MNNG/HOS cells were treated with SWs, DDP, and P2X7 siRNA, as indicated, and then apoptosis was detected using an annexin V–propidium iodide assay. SWs, shock waves.

increased the extracellular ATP concentration, suggesting that another mechanism may also exist, thus increasing the complexity of SW actions in osteosarcoma cells. Due to the crucial role of P2X7 in cancer progression, it is warranted to investigate whether SWs have an antitumor effect in metastatic osteosarcoma cells.

Although our results elucidated the cellular mechanisms of the effects of low-energy SWs on human osteosarcoma *in vitro*, the role of low-energy SWs *in vivo* needs to be validated. The complicated osteosarcoma microenvironment and immune features may weaken the in-vitro effects of low-energy SWs on the survival and metastasis of osteosarcoma cells. The correlation between P2X7 expression and the effectiveness of low-energy SWs on human osteosarcoma also warrants further investigation.

Although P2X7 has a dual role in cancer survival and growth, our results supported the pro-survival role of P2X7 in chemoresistant osteosarcoma cells. Our findings show that low-energy SWs significantly enhanced DDP cytotoxicity in osteosarcoma cells in the absence of P2X7. Low-energy SWs may mildly increase the extracellular ATP level, then inhibit the P2X7 cytotoxicity, and switch to maintain survival of osteosarcoma cells. This mechanism is consistent with the fact that low-energy SWs promoted osteosarcoma cell survival and proliferation (Fig. 1).

Conclusions

In conclusion, low-energy SWs promoted the survival and proliferation of human osteosarcoma MNNG/HOS cells, and this effect depended on the extracellular ATP receptor P2X7 via the Akt and mTOR pathway at the mRNA and protein levels, respectively. Furthermore, we also found that low-energy SWs enhanced the DDP cytotoxicity in the absence of P2X7. As a result, low-energy SWs may provide a novel therapeutic strategy for chemoresistant osteosarcoma patients and may be applied to treat metastatic osteosarcoma in the future.

Ethics statement

Ethics approval was not required for this experimental research.

Consent

Informed consent was not required for this experimental research.

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Author contribution

G.N. carried out the studies. W.J. drafted the manuscript and interpretated the data. T.Y. participated in the design of the study. B.Q. conceived of the study and participated in its design and coordination. All authors read and approved the final manuscript.

Conflicts of interest disclosure

The authors declare that they have no conflicts of interest.

Research registration unique identifying number (UIN)

Not applicable.

Guarantor

Bao-chang Qi.

Availability of data and materials

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Provenance and peer review

Our paper was not invited.

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