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Sonodynamic Treatment Combined with PEITC Effects on C6 Glioma Cells *In Vitro*

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Abstract

The aim of the study was hematoporphyrin monomethyl ether-mediated sonodynamic therapy combined with PEITC to kill C6 cells.

Methods: In the SDT+PEITC group, C6 cells received acoustic power treatment at an intensity of 0.5 W/cm2 and a frequency of 1 MHz, followed by incubated in 10 μM of the ITCs was used for 6 h. Tumor inhibition rate was assessed by MTT method and apoptosis rate was assessed by flow cytometry. Reactive Oxygen Species (ROS) and mitochondrial depolarization were detected by applying their respective methods. Additionally, cleavage of caspase-3 and caspase-9 was investigated through immunoblotting.

Results: In SDT+PEITC, the tumor suppression rate was significantly higher than that in the SDT or PEITC alone group. And, the apoptosis rate in the SDT+PEITC group was the highest. Additionally, the SDT+PEITC group exhibited higher levels of ROS generation and mitochondrial depolarization compared to the SDT or PEITC alone groups.

Conclusion: Based on our findings, it can be concluded that the combined treatment of SDT and PEITC, known as SDT+PEITC, exerts a potent cytotoxic effect on C6 glioma cells *in vitro* through synergistic mechanisms.

Keywords: C6 glioma cells; Apoptosis; SDT+PEITC (Sonodynamic Therapy combining with Phenethyl Isothiocyanate); Reactive Oxygen Species (ROS)

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Introduction

Based on ultrasound's good penetration, focus ability. Compared to electromagnetic modalities, such as laser beams, this advantage makes it more beneficial for treating deep-seated tumors invasively [1-3]. These techniques are therefore suitable for treating various types of cancer, although a study indicates that Sonodynamic Therapy (SDT) has an unsatisfactory delayed killing effect, thus suggesting the need for the integration of an alternative therapeutic approach [4-6].

As ROS are important for killing cancer cells, sonosensitizers produce excessive ROS locally by emitting waves of ultrasound into tumor tissues, then induce apoptosis/necrosis of cells [7]. As a result, ROS is considered a double-edged sword, i.e., the appropriate level of ROS plays a key role in maintaining homeostasis of cells, as well as mitosis, migration, and transformation [7-9]. The role of ROS produced by sonosensitizers in cancer therapy is driven by sites-specific overproduction of ROS in tumors. As a consequence, SDT's therapeutic potential in tumor tissues depends primarily on the ability of sonosensitizers to produce ROS [9]. In order to regulate intracellular reactive oxygen species levels and maintain intracellular homeostasis, there are corresponding resistance mechanisms to oxidative stress generated by the action of SDT, i.e., Intracellular levels of Reactive Oxygen Species (ROS) are controlled by intracellular molecules with antioxidant properties, such as GSH (reduced Glutathione) or HO-1 (Heme Oxygenase-1) [10]. It was reported by Musaogullari et al. [11] that inhibition of antioxidant molecules enhances the accumulation of intracellular ROS. Jia et al. [12] reported that PEITC effectively inhibits reactive oxygen species scavengers, thereby synergistically enhancing the cytotoxicity of reactive oxygen species, and enhances the cytotoxicity of BMN 673 by excess ROS-induced DNA damage and apoptosis.

Therefore, in the current study, we observed that PEITC combined with SDT activated HMME killing of C6 glioma cells. We simultaneously investigate the interplay between the two treatment

modalities.

Materials and Methods

Cell cultivation

Rat C6 glioma cells were obtained from the Institute of Neurosurgery, Harbin Medical University. A humidified incubator (Nuaire, Plymouth, MN) was used to maintain C6 cells in Roswell Park Memorial Institute-1640 medium (Hyclone Lab, Logan, UT, USA) at 37°C with 5% CO_2. The following experiments were conducted on cells in the exponential phase of growth.

PEITC and sonodynamic treatment

All operations were carried out at 37°C. By centrifugation, exponentially growing cells were resuspended (10^6 cells/ml) in serum free 1,640 medium for 4 h as well as incubated with HMME. A multi-functional ultrasound device (1 MHz) (Tianshi Technologies Limited) was used in experiments to demonstrate the efficacy of HMME at 10 ug/ml in cells. Stainless-steel ball radiometers (diameter 0.32 cm) were used to measure ultrasonic intensities (0.5 W/cm^2). Further details are provided in Li. Followed by incubated in 10 μM of the ITCs was used for 6 h in the following experiments.

The experiments were divided into different groups. In the control group, neither SDT nor PEITC was used on the cells. Cells were treated with 10 μM of the ITCs in the PEITC group. The cells were treated with HMME (10 g/ml) and ultrasonic pulses in the SDT group and low-intensity sonication at 0.5 W/cm^2. The SDT+PEITC group received SDT with 10 m of the ITCs.

Cell survival assay

After treatment, cells were washed and re-suspended in RPMI-1640 medium, followed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium chloride), which measures cell viability. A mixture of 20 ul MTT (5 mg/ml) and 100 ul DMSO was added to each well, followed by 100 ul DMSO to dissolve the formed formazan crystals after four hours. A reader for enzyme-linked immunosorbent assays was used (SpectraMax; Molecular 105 Devices, Sunnyvale, CA) for reading absorbance at 490 nm.

The survival rate was calculated by the following formula:

Inhibition rate (%) = (1- Optical Density (OD) treatment group/ Optical Density (OD) control group × 100%)

Cells were examined for apoptosis or necrosis

After re-incubation in the dark for up to four hours and washing twice with Phosphate Buffered Saline (PBS). A cell suspension of 100 μl was mixed with 5 μl of Annexin V FITC (Fluorescein Isothiocyanate, BD, Franklin Lakes, NJ) after adjusting cell density to 1×10^6 cells/ml. After gentle vortexing, the mixture was incubated for 15 min at room temperature (25°C) in the dark.

The number of apoptosis cells was measured using a flow cytometer (BD, Franklin Lakes, NJ, USA) after adding 400 μl of binding buffer.

Measurement of ROS generation

The detection of SDT-mediated ROS production was performed using 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) (Beyotime Institute of Biotechnology, Shanghai, China).

Incubation at 37°C for 20 min in the dark after adding DCFH-DA to the cell suspension at a concentration of 10 mol/L.

Using a fluorescent microplate reader (FLx800, BioTeK, Winooski, VT, USA) we measured the fluorescent dichlorofluorescein produced by oxidizing DCFH-DA.

Western blot assessing caspase-3,9 activation

The activation of caspase-3,9 was detected by Western blotting after the cells were re-incubated in the dark for up to 4 h after treatment.

To examine caspase-3,9 activation, cells of different groups were separately washed, collected and homogenized in a lysis buffer (10 mM Tris-HCl, pH 8, 0.32 mM sucrose, 5 mM EDTA, 2 mM DTT, 1 mM phenylmethyl sulfonyl fluoride, and 1% Triton X-100), and followed by centrifuged. Each group of proteins was electrophoresed on polyacrylamide gel (12%), and the gel-separated proteins were transferred to nitrocellulose membranes (Santa Cruz Biotechnology) and the membranes were probed with primary antibodies overnight at 4°C.

The targeted proteins were immunostained by different antibodies. Among the antibodies presented were: Anti-actin and anti-cleavage caspase-3,9 from Santa company. Following probing, the membranes were washed three times, and then incubated with the alkaline phosphatase-conjugated secondary antibodies (sigma) for 1 h at room temperature before visualization by using a chemiluminescence detection kit (sigma).

Statistical analysis

Statistical evaluation was performed with *t* test by the analytical tools of SPSS. Software system. Data are presented as mean values and Standard Errors of the mean (SE). The significance level was considered to be less than 0.05.

Results

Inhibition of cell growth in SDT+PEITC compared with SDT or PEITC

The growth inhibition rate of C6 glioma cells was determined by MTT assay in SDT combining with 10 μM of the ITCs. SDT or PEITC alone inhibited cell growth, but the inhibition rate was much higher in SDT+PEITC groups than in SDT or PEITC alone. In other words, our data indicated that SDT+PEITC could induce a synergetic killing effect on C6 cells (Figure 1).

SDT+PEITC-induced apoptosis

The flow cytometer assay showed marked changes in cell profile after SDT+PEITC. That SDT combining with PEITC induced C6 cells apoptosis or necrosis were evaluated by the flow cytometer. In SDT+PEITC groups, cells apoptosis rate was raising. Results suggested that SDT+PEITC could induce the most cells apoptosis by SDT combining with PEITC. The apoptotic rate was about 52.6 ± 4.54%. Among the groups, SDT+PEITC showed the highest rate of apoptosis in C6 glioma cells ($P<0.05$), while SDT or PEITC group was higher comparing with control ($P<0.01$), but lower than SDT+PEITC group. ($P<0.05$). HMME- mediated SDT+PEITC also increased the apoptotic rate C6 cells in some certain condition (Figure 2, 3).

The effect of Reactive Oxygen Species (ROS) on SDT-induced cell killing

To demonstrate the role of Reactive Oxygen Species (ROS) in SDT+PEITC induced cell death, ROS production was confirmed by burning DCFH-DA.

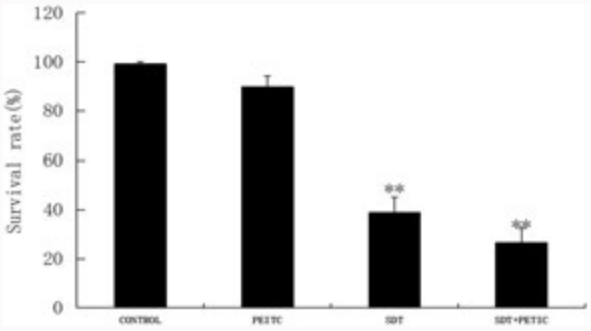

Figure 1: Percent of the growth inhibition rate of rat C6 glioma cells measured by a MTT assay after different treatment. Data represent mean ± SE (n=6). ** P<0.01 *vs.* control.

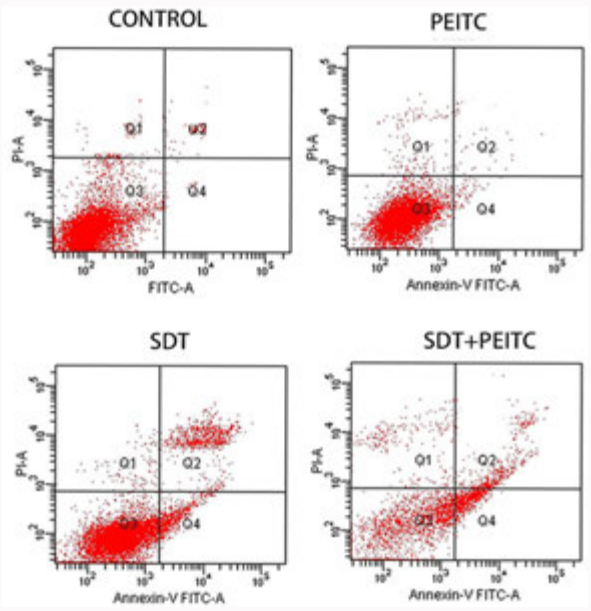

Figure 2: Apoptosis and necrosis measured in rat C6 glioma cells 4 hours after different treatment as measured by flow cytometry of annexin V-PI treated cells.

Based on the results, the synergistic effect of SDT+PEITC generated more ROS than either SDT or PEITC alone (Figure 4).

The effect of Mitochondrial depolarization by Reactive Oxygen Species (ROS)

To demonstrate the effect of Mitochondrial depolarization in SDT+PEITC induced cells killing, ROS causes Mitochondrial depolarization of C6 cells was confirmed by the DiOC6(3). In this study, synergistic effects of SDT + PEITC produced more mitochondrial depolarization than SDT or PEITC alone ($p<0.01$) (Figure 5).

SDT+PEITC can induce caspase-3,9 activation

As shown by the Western blot analyses of cytosolic extracts, SDT+PEITC could induce caspase-3,9 cleavage. Band intensity quantitation measurements showed that the caspase-3,9 activation markedly magnified than SDT or PEITC group alone (Figure 6).

Discussion

Ultrasound at specific wavelengths, they emit toxic reactive oxygen species. As a result, SDT can selectively kill cancer cells at

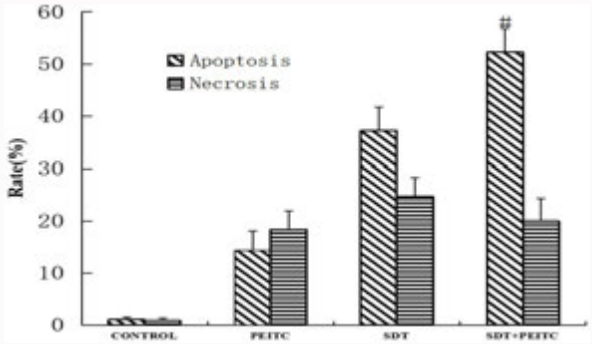

Figure 3: Data represent mean ± SE (n=6). #P<0.05 *vs.* the other groups.

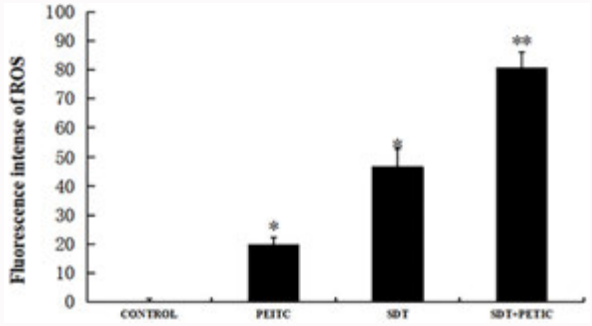

Figure 4: ROS production as indicated by DCFH fluorescence in different groups. *P<0.05 *vs.* control, **P<0.01 *vs.* control.

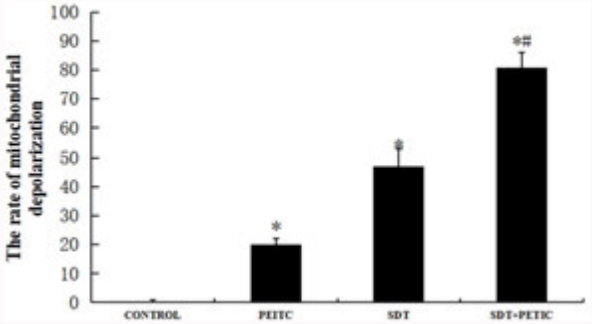

Figure 5: Mitochondrial depolarization as indicated by DiOC6(3) fluorescence in different groups. Fluorescent dichlorofluorescein generated from the oxidation of DiOC6(3) was measured by a Flow cytometer. The rate of mitochondrial depolarization were represent, data represent mean ± SE (n=6). *P<0.05 *vs.* control and PEITC group. # P<0.01 *vs.* he other groups.

ultrasonic sites, resulting in fewer side effects [13-16]. But SDT also suffers from several drawbacks, such as rapid clearance, and lack of later killing which lead to an insufficient therapeutic effect [17-19].

In the present work, Remarkable cell killing was observed SDT+PEITC *in vitro*. The cell damaging rate was much higher than SDT or PEITC alone (Figure 1). Experimental results indicated that SDT+PEITC could effectively kill C6 glioma cells *in vitro*. Almost 73% of tumor cells were killed by SDT+PEITC.

Apoptosis is one of the major pathways of tumor cell death. Many new therapeutic methods of tumor should target apoptosis [20]. Our experiment examined the effectiveness and mechanism of SDT+PEITC on inducing apoptosis in C6 glioma cells.

Remarkable cell apoptosis was observed in SDT+PEITC *in vitro*.

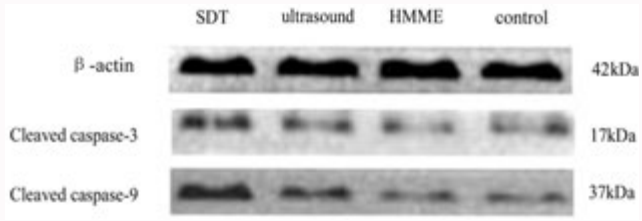

Figure 6: Caspase-3 and caspase-9 cleavage in C6 cells after HMME-SPDT. Representative western blots for the expression of Caspase-3 and caspase-9 cleavage in different groups.

The cell apoptosis rate was much higher than SDT or PEITC alone (Figure 2, 3). *In vitro* experiments showed that SDT+PEITC can induce outstandingly apoptosis in C6 glioma cells. SDT or PEITC alone could also induce small glioma cells apoptosis, but significantly lower than SDT+PEITC group at 6 h. So, it is indicated that SDT and PEITC had synergic effect on inducing tumor cells apoptosis.

Some researches had demonstrated that ROS could be generated followed by SDT [3-5]. Oxidative stress was reported to be involved in the dysfunction of mitochondria which was marked with the mitochondrial transmembrane potential collapses [21-23], and the mitochondria release proapoptotic factors such as cytochrome c, which is essential to the formation of apoptosis, which ultimately cleave pro-caspase-3 to form active caspase-3. Therefore, we detected that SDT+PEITC could increase ROS level *in vitro* (Figure 4). SDT or PEITC alone also causes generation of over ROS. But ROS in SDT or PEITC group was much lower than that in SDT+PEITC group. Our experiment also demonstrated that SDT+PEITC could magnify release of cleavaged-caspase-9 and caspase-3 in cytoplasm (Figure 5, 6). The change in SDT or PEITC group was not apparent than SDT+PEITC. These results manifested Mitochondria-dependent caspase pathway may be one of channel of SDT+PEITC induce C6 glioma cells apoptosis. As a result, the mitochondria release proapoptotic factors such as cleavaged-caspase-3, which is essential for apoptosis to occur.

This is the important reason for favorable antitumor effects. These GSH conjugates are exported out of the cells through membrane-bound transporter proteins. Given the fact that GSH plays critical roles in maintaining redox homeostasis in biological systems [24], Moreover, the high Glutathione (GSH) level of tumor cells can cause the generated ROS to be consumed during SDT, significantly compromising the efficacy of SDT. We hypothesized that the high GSH level of tumor cells could compromise the efficacy of SDT and that depleting intracellular GSH of tumor cells by PEITC could boost the efficacy of SDT. The conjugation of PEITC with intracellular GSH and the subsequent removal of the conjugate result in the depletion of intracellular GSH [25]. PEITC can deplete GSH efficiently and selectively and thus eradicates cancer cells while sparing normal cells [25-27].

This *in vitro* study may be preliminary and the treatment effectiveness needs to be verified *in vivo* in the future, but it strongly suggests that SDT+PEITC might be a promising method for the treatment of glioma.

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References

1. Kessel D, Jeffers R, Fowlkes JB, Cain C. Effects of sonodynamic and photodynamic treatment on cellular thiol levels. J Photochem Photobiol B. 1996;32(1-2):103-6.
2. Worthington AE, Thompson J, Rauth AM, Hunt JW. Mechanism of ultrasound enhanced porphyrin cytotoxicity. Part I: A search for free radical effects. Ultrasound Med Biol. 1997;23(7):1095-105.
3. Yumita N, Umemura S. Sonodynamic therapy with photofrin II on AH130 solid tumor. Pharmacokinetics, tissue distribution and sonodynamic antitumoral efficacy of photofrin II. Cancer Chemother Pharmacol. 2003;51(2):174-8.
4. Rosenthal I, Sostaric JZ, Riesz P. Sonodynamic therapy - A review of the synergistic effects of drugs and ultrasound. Ultrason Sonochem. 2004;11(6):349-63.
5. Wang P, Wang X, Zhang K, Gao K, Song M, Liu Q. The spectroscopy analyses of PpIX by ultrasound irradiation and its sonotoxicity *in vitro*. Ultrasonics. 2013;53(5):935-42.
6. Nomikou N, Sterrett C, Arthur C, McCaughan B, Callan JF, McHale AP. The effects of ultrasound and light on indocyanine-green-treated tumor cells and tissues. Chem Med Chem. 2012;7(8):1465-71.
7. Zhou D, Shao L, Spitz DR. Reactive oxygen species in normal and tumor stem cells. Adv Cancer Res. 2014;122:1-67.
8. Fehér A, Otvös K, Pasternak TP, Szandtner AP. The involvement of Reactive Oxygen Species (ROS) in the cell cycle activation (G(0)-to-G(1) transition) of plant cells. Plant Signal. Behav. 2008;3(10):823-6.
9. Zhang Y, Zhang X, Yang H, Yu L, Xu Y, Sharma A, et al. Advanced biotechnology-assisted precise sonodynamic therapy. Chem Soc Rev. 2021;50(20):11227-48.
10. Niu B, Liao K, Zhou Y, Wen T, Quan G, Pan X, et al. Application of glutathione depletion in cancer therapy: Enhanced ROS-based therapy, ferroptosis, and chemotherapy. Biomaterials. 2021:277:121110.
11. Musaogullari A, Mandato A, Chai YC. Role of glutathione depletion and reactive oxygen species generation on caspase-3 activation: A study with the kinase inhibitor staurosporine. Front Physiol. 2020;11:998.
12. Jia Y, Wang M, Sang X, Liu P, Gao J, Jiang K, et al. Phenethyl isothiocyanate enhances the cytotoxic effects of PARP inhibitors in high-grade serous ovarian cancer cells. Front Oncol. 2022;11:812264.
13. D'Ammando A, Raspagliesi L, Gionso M, Franzini A, Porto E, Di Meco F, et al. Sonodynamic therapy for the treatment of intracranial gliomas. J Clin Med. 2021;10(5):1101.
14. Araújo Martins Y, Zeferino Pavan T, Fonseca Vianna Lopez R. Sonodynamic therapy: Ultrasound parameters and *in vitro* experimental configurations. Int J Pharm. 2021;610:121243.
15. Mehta NH, Shah HA, D'Amico RS. Sonodynamic therapy and sonosensitizers for glioma treatment: A systematic qualitative review. World Neurosurg. 2023;178:60-8.
16. Wang L, Hu Y, Hao Y, Li L, Zheng C, Zhao H, et al. Tumor-targeting core-shell structured nanoparticles for drug procedural-controlled release and cancer sonodynamic combined therapy. J Control Release. 2018;286:74-84.
17. Li JH, Chen ZQ, Huang Z, Zhan Q, Ren FB, Liu JY, et al. *In vitro* study of low intensity ultrasound combined with different doses of PDT: Effects on C6 glioma cells. Oncol Lett. 2013;5(2):702-6.
18. Tserkovsky DA, Alexandrova EN, Chalau VN, Istomin YP. Effects of

combined sonodynamic and photodynamic therapies with photolon on a glioma C6 tumor model. Exp Oncol. 2012;34(4):332-5.

19. Hu X, Zhang YS, Liu YC, Wang N, Zeng XT, Zhang LL. Emerging photodynamic/sonodynamic therapies for urological cancers: Progress and challenges. J Nanobiotechnology. 2022;20(1):437.
20. Valter K, Zhivotovsky B, Gogvadze V. Cell death-based treatment of neuroblastoma. Cell Death Dis. 2018;9(2):113.
21. Rogalska A, Koceva-Chyła A, Jóźwiak Z. Aclarubicin-induced ROS generation and collapse of mitochondrial membrane potential in human cancer cell lines. Chem Biol Interact. 2008;176(1):58-70.
22. Das R, Roy A, Dutta N, Majumder HK. Reactive oxygen species and imbalance of calcium homeostasis contributes to curcumin induced programmed cell death in *Leishmania donovani*. Apoptosis. 2008;13(7):867-82.
23. Yang J, Wu LJ, Tashino S, Onodera S, Ikejima T. Critical roles of reactive oxygen species in mitochondrial permeability transition in mediating evodiamine-induced human melanoma A375-S2 cell apoptosis. Free Radic Res. 2007;41(10):99-108.
24. Hu H, Chen J, Yang H, Huang X, Wu H, Wu Y, et al. Potentiating photodynamic therapy of ICG-loaded nanoparticles by depleting GSH with PEITC. Nanoscale. 2019;11(13):6384-93.
25. Lv H, Zhen C, Liu J, Shang P. β-phenethyl isothiocyanate induces cell death in human osteosarcoma through altering iron metabolism, disturbing the redox balance, and activating the MAPK signaling pathway. Oxid Med Cell Longev. 2020;2020:5021983.
26. Hong YH, Uddin MH, Jo U, Kim B, Song J, Suh DH, et al. ROS accumulation by PEITC selectively kills ovarian cancer cells *via* UPR-mediated apoptosis. Front Oncol. 2015;5:167.
27. Lv HH, Zhen CX, Liu JY, Shang P. PEITC triggers multiple forms of cell death by GSH-iron-ROS regulation in K7M2 murine osteosarcoma cells. Acta Pharmacol Sin. 2020;41(8):1119-32.