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● *Original Contribution*

THE COMBINATION OF GLYCOLYTIC INHIBITOR 2-DEOXYGLUCOSE AND MICROBUBBLES INCREASES THE EFFECT OF 5-AMINOLEVULINIC ACID-SONODYNAMIC THERAPY IN LIVER CANCER CELLS

RUI XIE,* TONGYING XU,* JIUXIN ZHU,† XIAOLI WEI,* WENTING ZHU,* LONGMIN LI,* YUFENG WANG,* YU HAN,* JIANHUA ZHOU,* and YUXIAN BAI*

*Department of Digestive Internal Medicine & Photodynamic Therapy Center, Harbin Medical University Cancer Hospital, Harbin, China; and †Department of Pharmacology (the State-Province Key Laboratories of Biomedicine-Pharmaceutics of China, Key Laboratory of Cardiovascular Research, Ministry of Education), Harbin Medical University, Harbin, China

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Abstract—Sonodynamic therapy (SDT) overcomes the shortcoming of photodynamic therapy in the treatment of cancer. Previous studies indicated that the glycolysis inhibitor 2-deoxyglucose (2-DG) potentiated photodynamic therapy induced tumor cell death and microbubbles (MBs) improved the SDT performance. We hypothesized that the combination of 2-DG and MBs will increase the effect of 5-aminolevulinic acid (ALA)-SDT in HepG2 liver cancer cells. When cells were treated with 5-min ALA-SDT and 2-mmol/L 2-DG, the cell survival rate decreased to 73.0 ± 7.1% and 75.2 ± 7.9%, respectively. Furthermore, 2 mmol/L 2-DG increased 5-min ALA-SDT induced growth inhibition and augmented ALA-SDT induced cell apoptotic rate from 9.8 ± 0.7% to 17.4 ± 2.2%. In the combination group (2-DG and ALA-SDT group), HepG2 cells possessed typical apoptotic characters. 2-DG also increased ALA-SDT associated intracellular reactive oxygen species generation and loss of mitochondrial membrane potential. Moreover, SonoVue MBs had stimulatory function on cell viability inhibition, apoptosis, reactive oxygen species production and mitochondrial membrane potential loss for combination treatment. This study suggests a promising therapeutic strategy using a combination of 2-DG, MBs and ALA-SDT for treating liver cancer. (E-mail: xierui178@126.com) © **2017 World Federation for Ultrasound in Medicine & Biology.**

Key Words: **Liver cancer, Sonodynamic therapy, 5-aminolevulinic acid, 2-deoxyglucose, Microbubbles.**

INTRODUCTION

Liver cancer is the second leading cause of cancer death in the world (Ferlay et al. 2015). The 5-y survival rate in liver cancer is generally below 20% both in the developing and developed world (Allemani et al. 2015). The main treatments for liver cancer are surgical resection, transarterial chemoembolization, radiofrequency catheter ablation, percutaneous microwave coagulation therapy and targeted therapy. However, these treatment modalities show unsatisfactory results (Liu et al. 2014). High-intensity focused ultrasound can apply the option to combine with a low-morbidity local therapy in the treatment of hepatocellular carcinoma (Aubry et al. 2013). Thus, novel therapeutic methods using ultrasound for liver cancer are required.

Sonodynamic therapy (SDT) involves treatment of the target tissue with a sensitizer and ultrasound exposure to the target tissue (McHale et al. 2016). SDT overcomes the shortcomings of inadequate tissue penetration and invasive properties of the photodynamic therapy in cancer. Many of the sonosensitizers such as hematoporphyrin, Photofrin (Pinnacle Biologics, Chicago, IL, USA), chlorin-e6 and indocyanine green are used in SDT (McHale et al. 2016). 5-Aminolevulinic acid (ALA), a potent sonosensitizer, is converted into protoporphyrin IX (PpIX) and accumulates in the heme biosynthesis pathway of tumor cell mitochondria (Krammer and Plaetzer, 2008). Previous reports have demonstrated ALA-SDT produces antitumor effects on glioma (Jeong et al. 2012; Ohmura et al. 2011), tongue cancer (Song et al. 2011), osteosarcoma (Li et al. 2015b) and melanoma (Hu et al. 2015). In our previous study, we found that ALA-SDT induced pancreatic cancer cell killing *via* the mitochondrial pathway (Li et al. 2014).

Address correspondence to: Yuxian Bai, Department of Digestive Internal Medicine & Photodynamic Therapy Center, Harbin Medical University Cancer Hospital, Harbin 150081, China. E-mail: xierui178@126.com

Unlike normal cells, cancer cells usually exhibit an increased glucose uptake and glycolysis in the presence of oxygen, and this phenomenon is referred to as the *Warburg effect* or *aerobic glycolysis* (Devic 2016; Ngo et al. 2015). The glycolysis inhibitor 2-deoxyglucose (2-DG) induces oxidative stress and kills tumor cells (Shutt et al. 2010). Oxidative stress refers to increased intracellular reactive oxygen species (ROS) levels that cause damage to deoxyribonucleic acid (DNA), proteins and lipids (Schieber and Chandel 2014). Moreover, many reports confirmed that SDT induced the production of a high amount of intracellular ROS and subsequently generated direct cytotoxicity in malignant cells (Wan et al. 2016). Thus, metabolic inhibitors such as 2-DG seem to be a rational choice as an SDT supplement to induce more ROS and kill tumors.

Gas-filled microbubbles (MBs) are widely used as ultrasound contrast agents to improve the characterization and detection of malignant lesions (Dijkmans et al. 2004; Kiessling et al. 2012). Acoustic cavitation of MBs has been recognized to play a vital part in the cell membrane destruction caused by ultrasound (sonoporation) (Zhou et al. 2012). MBs have been proposed as a new promising tool for gene or drug delivery and they are also able to improve the effect of chemotherapy (Yang et al. 2014; Yoon et al. 2014). In addition, SDT is mediated through sonosensitizers combining with ultrasound-induced cavitation to produce free radicals that destroy tumors (Wood and Sehgal 2015). It is reported that in comparison with the sonosensitizer alone, MB and sonosensitizer conjugates enhance cytotoxicity in cancer cells and inhibit tumor growth (Nomikou et al. 2012). Similarly, MB-enhanced antitumor effects in sinoporphyrin sodium or hematoporphyrin monomethyl ether mediated SDT (Ruan et al. 2013; Wang et al. 2015a). In addition, SonoVue, a contrast material involving sulphur hexafluoride MBs (Lindner 2004), is indicated for contrast-enhanced ultrasound imaging of the liver disease (Nicolau et al. 2004; Westwood et al. 2013; Yang et al. 2016). In this study, we mainly focused on the combined effect of 2-DG and SonoVue MBs on ALA-SDT.

MATERIALS AND METHODS

Experiment design

Human hepatic cancer cell line HepG2 cells, served as the experiment model system (Fig. 1). Cells were incubated with 1 mmol/L of ALA. Intracellular PpIX accumulation and subcellular localization of PpIX were detected using fluorescence spectrometer and confocal microscope, respectively, to obtain the optimal incubation time of ALA. After different treatment, we tested cell viability, cell apoptosis, cell ultrastructure, ROS generation and mitochondrial membrane potential (MMP) loss.

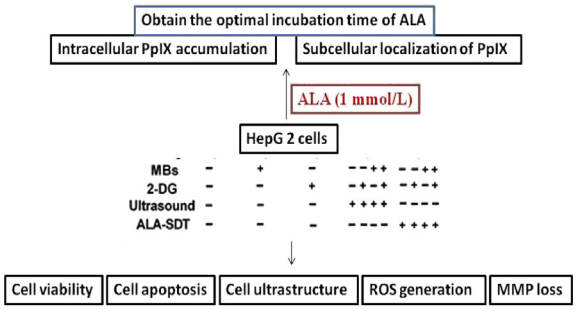

Fig. 1. The experimental workflow. HepG2 cells were incubated with 1 mmol/L of ALA; intracellular PpIX accumulation and subcellular localization of PpIX were detected to obtain the optimal incubation time of ALA. After various types of treatment, cell viability, cell apoptosis, cell ultrastructure, ROS generation and MMP loss were tested. ALA = 5-aminolevulinic acid; PpIX = protoporphyrin IX; ROS = reactive oxygen species; MMP = mitochondrial membrane potential.

Reagents

The ALA and Annexin V-FITC Apoptosis Detection Kit were obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum and RPMI 1640 were purchased from Hyclone Laboratories (Logan, UT, USA). 2′,7′-dichlorofluorescein diacetate (DCFH-DA) was supplied by Applygen Technologies Inc. (Beijing, PR China). MitoProbe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), a Cell Counting Kit-8 (CCK-8) and Trysin-EDTA Solution were from Beyotime Biotechnology (Haimen, Jiangsu, China). Sulphur hexafluoride MBs were SonoVue (Bracco Suisse SA, Geneva, Switzerland).

Cell culture and ALA treatment

Human hepatic cancer cell line HepG2 cells were purchased from American Type Culture Collection (ATCC, Manassass, VA, USA). HepG2 cells were cultured in RPMI-1640 containing 10% fetal bovine serum, 0.1 mg/mL streptomycin and 100 U/mL penicillin. The HepG2 cells were maintained in humidified air containing 5% CO_2 at 37°C. When tumor cells reached the logarithmic growth phase, the experiments were carried out.

Cells adhered to the culture dishes were co-incubated with ALA in the dark. To obtain the suspension cells, RPMI 1640 was removed and residual serum was eliminated using phosphate-buffered saline (PBS). Trypsin-ethylenediaminetraacetic acid (EDTA) solution was slowly added to cover the cell monolayer, then culture media containing fetal bovine serum was used to inactivate the trypsin.

Intracellular PpIX accumulation

To detect the intracellular kinetics of ALA-PpIX, the cells were incubated with 1-mmol/L ALA at various