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Dihydroartemisinin increases radiosensitivity of A549 lung cancer cells

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ABSTRACT

Background: Radiotherapy is the gold standard in the treatment of lung cancer. However, the radiosensitization of cancerous cells requires further improvement. Here, we investigated the effect of dihydroartemisinin (DHA) on the radiosensitization of non-small cell lung cancer (NSCLC) cells. ***Methods:*** Cell proliferation and cell cycle assays were carried out using A549 cells exposed to DHA. The effect of DHA on the radiosensitization of cells was investigated by clonogenic cell survival assay and apoptosis assay. Polymerase chain reaction and western blotting were used to quantify GSK-3β level. ***Results:*** DHA significantly inhibited cell proliferation from 24 to 96 h. Cells treated with DHA had a significantly decrease G1 phase but significantly increase S and G2/M phases compared with untreated and vehicle-treated cells. The number of viable DHA-treated cell colonies was significantly lower than that of untreated or vehicle-treated cells. The percentage of early apoptotic and necrotic cells was significantly higher for cells exposed to DHA and irradiation. GSK-3β expression was significantly higher in cells exposed to DHA and irradiation than that in untreated cells, indicating that DHA may enhances the radiosensitization of cells through the activation of GSK-3β. ***Conclusions:*** Our results demonstrate that DHA can increase the radiosensitivity of A549 cells, suggested its potential use to sensitize tumors to radiation therapy in NSCLC. The expressions of GSK-3β were induced by DHA in cells exposed to irradiation, indicating GSK-3β may paly important role in the radiosensitization mechanism of DHA.

Keywords: *Dihydroartemisinin, Non-small cell lung cancer, A549 cells, Radiosensitization, Glycogen synthase kinase-3beta.*

INTRODUCTION

Lung cancer is one of the most common types of cancer worldwide, with an estimated 2.1 million new diagnoses and 1.8 million deaths each year [1]. Among the different types of lung cancer, non-small cell lung cancer (NSCLC) constitutes approximately 85% of all lung cancers [2]. Although new treatments, such as immunotherapy and targeted therapy, have been shown to improve the prognostic outcome, radiotherapy remains the gold standard in the treatment of early stage and advanced NSCLC [3-5]. However, improvements are needed to enhance the radiosensitization of cancerous cells.

Artemisinin is a sesquiterpene lactone derived from *Artemisia annua*, a traditional Chinese medicinal plant [6]. Dihydroartemisinin (DHA), an active metabolite of artemisinin, is an antimalarial drug that has recently been shown to exhibit potent anti-cancer effects [7-9]. For instance, DHA can induce A549 cell apoptosis [10]. We previously demonstrated that DHA can inhibit the proliferation of cells, and resulted in cell cycle arrest in NSCLC [11]. Other studies have reported that artemisinin, artesunate, and DHA are toxic to radiation-resistant human breast

cancer cells and drug-resistant human NSCLC cells, particularly when the cells are pretreated with transferrin, which increases the intracellular $Fe2^{+}$ level [12, 13]. Here, we hypothesized that DHA may improve the radiosensitivity of A549 cells. Glycogen Synthase Kinase-3 beta (GSK-3β), a serine/threonine protein kinase, regulates radiosensitivity of tumors in several types of cancers [14, 15].

Thus, we hypothesized that DHA may enhance the radiosensitization of A549 cells through GSK-3β. In this study, we explored the effect of DHA on the radiosensitization of A549 cells and assess the mechanism of action, aiming to provide ample evidence for potential use of DHA in sensitizing NSCLC to radiation therapy.

MATERIALS AND METHODS

Cell and culture

The non-small cell lung cancer cell line A549 was obtained from the Cell Bank of the Committee on the Type Culture Collection of the Chinese Academic of Science (CCTCC, Shanghai, China). Cells were seeded in flasks and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Gibco), 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. Cells were exposed to radiation, which was generated with a linear accelerator (UNIQUE; Varian, Palo Alto, CA, USA).

Determination of the IC10 of DHA

A549 cells were seeded in 96-well plates at 5.0 ´ 10^4/well, incubated for 9 h, and then exposed to different concentrations of DHA for three days. Cells of the blank group were treated with DMEM, and those of the control group were treated with DMEM containing 1% dimethyl sulfoxide (vehicle). The absorbance at wavelength 450 mm was measured after three days.

Cell proliferation assay

Cell proliferation was assessed with the Cell Count Kit-8 (CCK-8 Beyotime, China), according to the manufacturer's instructions. A549 cells were seeded in 96-well plates at 5.0 ´ 10^4/well and incubated for four days. Cells of the blank group were treated with DMEM, and those of the control group were treated with DMEM containing 1% DMSO. The absorbance at wavelength 450 mm was measured from days 1 to 4.

Cell cycle assay

The cell cycle was analyzed by flow cytometry (FCM) with propidium iodide staining. Both attached and detached cells were collected by trypsin digestion, centrifuged at low speed, washed with ice-cold phosphate buffered saline (PBS), and then fixed in ice-cold 70% ethanol overnight. Cells were collected by a brief centrifugation step and resuspended in PBS, after which they were treated with RNase A, stained with propidium iodide for 1 h at room temperature, and analyzed by FCM.

Clonogenic cell survival assay

A549 cells were seeded in 6-well plates at 200 ´ 10^4/well and cultured in the presence or absence of DHA (DMEM or vehicle) for 6 h. Thereafter, DHA-treated and untreated cells were exposed to 2-Gy irradiation. Cells were incubated for 14 days, fixed with ethanol, and then stained with 0.1% crystal violet. Colonies with more than 50 cells were counted under a Leica DM4B microscope (Wetzlar, Germany).

Apoptosis assay

A549 cells were seeded in 6-well plates at 5 ´ 10^5/well and cultured in the presence or absence (DMEM or vehicle) of DHA for 6 h. Thereafter, DHA-treated and untreated cells were exposed to 2-Gy irradiation. Cells were harvested and washed twice with PBS. Cell viability was assessed with the Annexin V-PE Apoptosis Kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions. The samples were immediately analyzed by FCM.

Quantitative PT-PCR

Total RNA was reverse transcribed into cDNA with the One-Step SYBR PrimeScript RT-PCR Kit

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II (Takara Biotechnology, Dalian, China), and qRT-PCR was performed using SYBR Premix Ex *Taq* (Takara Biotechnology) according to the manufacturer's instructions. The primers for GSK-3β were 5′–ACTGTGTAGCCGTCTGCTGGAG–3′ (forward) and 5′–CAGGTGGAGTTGGAAGCTGATGC–3′ (reverse). The primers for GAPDH were 5′–ACCTGACCTGCCGTCTAGAA–3′ (forward) and 5′–TCCACCACCCTGTTGCTGTA–3′ (reverse). The results were analyzed by the $^{\Delta\Delta}$Ct method. Each experiment was repeated three times.

Antibodies and western blotting

The antibodies used in this study were GSK-3β (Cell Signaling Technology, Danvers, MA, USA) and β-ACTIN (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Proteins were extracted from cells with RIPA buffer (Beyotime Institute of Biotechnology, Nantong, China), separated on a sodium dodecyl sulfate polyacrylamide gel, and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). Antibody-–antigen complexes were detected using enhanced chemiluminescence reagents (Advansta, Menlo Park, CA, USA). The blots were scanned with the ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, USA) and analyzed with the Image Lab Software (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All data were analyzed using SPSS 21.0 Software (SPSS Inc., Chicago, IL, USA). Statistical data are presented as the mean ± standard deviation (SD). Comparisons of continuous variables between two groups were performed using Student's *t*-test. Univariate analysis of variance was used for comparisons among three or more groups. *P*-values less than 0.05 were considered statistically significant.

RESULTS

Determination of the IC10 of DHA

To determine the IC10 of DHA, A549 cells were treated with different concentrations DHA for three days, as shown in table 1. The IC10 of DHA was 23.47 μM.

Table 1. The effect of different concentrations of DHA on A549 cells.

Concentration ($\mu mol \cdot L^{-1}$)	OD_{450}	Inhibitory rate (%)
300	0.19±0.06	100.00
250	0.27±0.13	92.17
200	0.38±0.09	83.15
150	0.45±0.15	70.67
100	0.56±0.10	58.96
75	0.64±0.14	47.13
60	0.69±0.12	36.24
45	0.72±0.13	23.36
30	0.78±0.15	15.87
15	0.81±0.16	9.16

DHA inhibits the proliferation of A549 cells

A549 cells were treated with 23 μM DHA for 96 h, and the effect of DHA on the proliferation of cells was determined with the CCK-8 Kit. As shown in figure 1, DHA significantly inhibited cell proliferation from 24 to 96 h compared with untreated and vehicle-treated cells ($p < 0.05$).

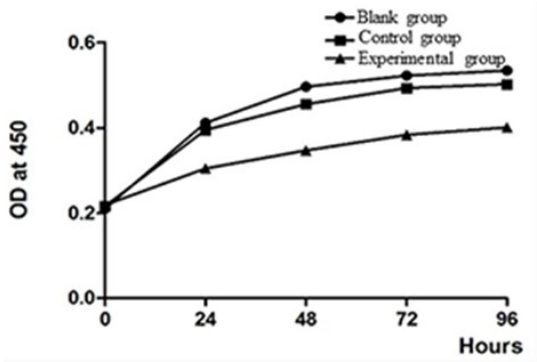

Figure 1. The influence of dihydroartemisinin on the proliferation of A549 cells was detected. DHA significantly inhibited A549 cell proliferationrom from the 24th hour to the 96th hour.

DHA induces cell cycle arrest in A549 cells

To examine the effect of DHA on the cell cycle, A549 cells were treated with 23 μM of DHA for 48 h. As shown in figure 2, cells treated with DHA had a significantly decrease G1 phase, but significantly increase S and G2/M phases compared with untreated and vehicle-treated

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cells ($p < 0.05$). These findings indicate that DHA arrested A549 cells at the S phase and the G2/M phase.

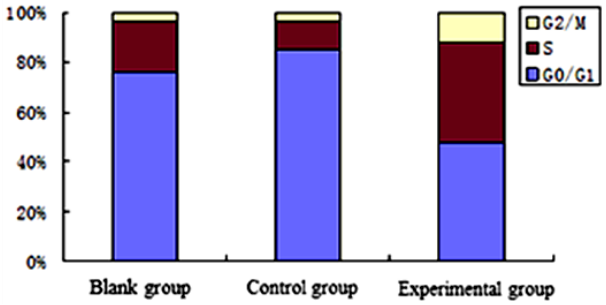


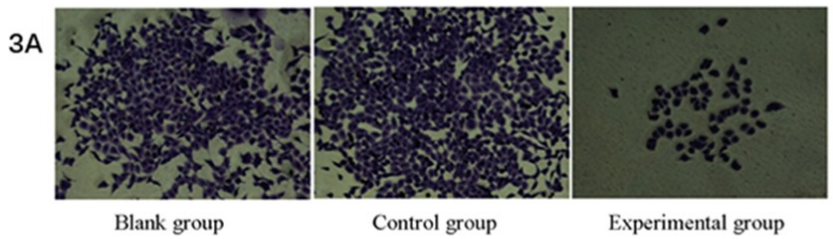
Figure 2. Dihydroartemisinin induced cell cycle arrest in A549 cells. Cells treated with DHA showed a decrease in G1 phase and an increase in both S phase and G2/M phase

DHA enhances the radiosensitization of A549 cells

To examine the effect of DHA on the radiosensitization of A549 cells, both clonogenic cell survival and apoptosis assays were performed. As shown in figure 3A, the number of viable DHA-treated cell colonies was significantly lower than that of untreated or vehicle-treated cells ($p < 0.05$). As shown in figure 3B, the percentage of early apoptotic and necrotic cells was significantly higher for cells exposed to DHA and irradiation than that for untreated or vehicle-treated cells ($p < 0.05$). These findings indicate that DHA increased the radiosensitivity of A549 cells.

DHA enhances the radiosensitization of A549 cells by activating GSK-3β

GSK-3β expression was examined in untreated A549 cells, cells exposed to irradiation, and cells exposed to both DHA and irradiation. As shown in Figure 4A, the GSK-3β mRNA level was significantly higher in cells exposed to both DHA and irradiation than that in untreated cells or those exposed to irradiation only ($p < 0.05$), consistent with the results of western blotting (figure 4B). These findings speculate DHA enhanced the radiosensitization of A529 cells through GSK-3β activation.



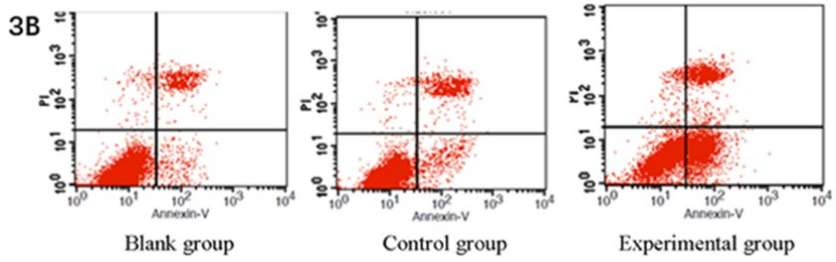


Figure 3. In clone formation experiment, dihydroartemisinin increased A549 radiation sensitivity (magnification in 100) **(3A).** Dihydroartemisinin increased the apoptosis of A549 cells after radiotherapy **(3B)**.

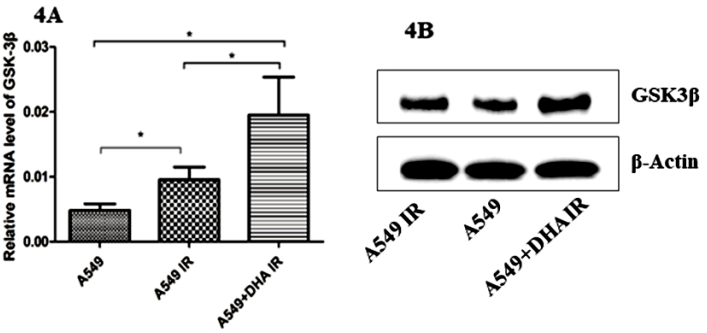


Figure 4. The expression of GSK3β was significantly higher in the experimental group (A549 cells treated with both DHA and irritation) among the three groups in both mRNA level **(4A)** and protein level **(4B)** ($p<0.05$).

DISCUSSION

Artemisinin and its derivatives (i.e., DHA) have been one of the drugs subject to drug repurposing with promising benefits in the field of oncology. The artemisinin-type drugs have been closely combined with various cancer therapies, such as standard chemotherapy, radiotherapy and photodynamic therapy [16]. Artemesinin has been tested as a radiosensitizer in glioblastoma cells [17], and Hela cell lines [18], and A549 lung cancer cells [19]. DHA has been studied as a radiosensitizer in glioblastoma [20], and cervical cancer[21], and lung cancer cell lines[22]. These studies have shown the radiosensitizing effects of artemisinin and dihydroartemesinin, with induction of apoptosis and G2/M cell cycle arrest. The current study has confirmed these findings in addition it has shown S phase accumulation and growth arrest. Such findings suggest that dihydroartemisinin may be a potential radiotherapy sensitization in non-small cell lung cancer.

Furthermore, to examine the underlying radiosensitization mechanism of DHA in A549 cells, we analyzed the expression of GSK-3β. GSK -3β, a multifunctional serine/threonine kinase, had been initially identified as a key regulator of insulin-dependent glycogen synthesis. GSK-3β interaction with various proteins; for example, GSK-3β stabilizes or activates mouse double minute 2 homolog (MDM2) and destabilizes or inactivates activator protein 1 (AP-1)[23]. Also, GSK-3β is activated by various pathways, including the PI3K/Akt and Wnt pathways [24-26]. Therefore, GSK-3β regulates a diverse array of cellular processes including proliferation, differentiation, motility and survival [27]. Consequently, the role of GSK3β in tumorigenesis and cancer progression remains controversial. It may function as a tumor suppressor for certain types of tumors [27, 28], but promotes growth and development for some others [29-31].

Although, previous studies have shown that GSK-3β expression is associated with NSCLC differentiation, and patients with GSK-3β-negative tumors had a better prognosis [31, 32]; on the other hand, studies have also demonstrated that GSK-3β inhibits autophagy [33, 34], which facilitates the removal of unwanted mitochondria, thereby decreasing the radiosensitivity of cancerous cells and protecting them from the effects of irradiation [35]. Ren J *et al.* has proved that GSK-3β can enhance the radiosensitivity of NSCLC cells, both in GSK-3β-high A549 cell line and in GSK-3β-low H460 cell line [32]. In our study, we found that GSK-3β level was significantly higher in cells treated with DHA than that in untreated or vehicle-treated cells, indicating DHA induced GSK-3β expression and increased the radiosensitivity of cells.

However, more studies are still needed to explore the radiosensitization mechanism of DHA. On the other hand, Li X *et al.* have investigated the cytotoxicity of artemisinin and artesunate on A549 cell line and on human bronchial epithelium. They demonstrated a cytotoxic effect on both cell lines but higher in A549 cell line [36]. Our current study only investigates the radiosensitization on lung cancer cell lines. Further studies are still needed to clear the cytotoxicity of DHA on non-cancer tissues with and without radiotherapy.

CONCLUSION

In conclusion, DHA can inhibit the proliferation of A549 cells and arrest cell cycle. DHA also increased the radiosensitivity of A549 cells, suggested its potential use to sensitize tumors to radiation therapy in NSCLC. In addition, DHA induced the expression of GSK-3β in cells exposed to irradiation, indicating GSK-3β may paly important role in the radiosensitization mechanism of DHA.

Authors' contributions

Kui Liao and Juan Li contributed to concept and design; Kui Liao provided administrative support; Juan Li provided study materials; Xinyou Li collected and assembled data; Shunlong Wu analyzed data; all authors wrote the manuscript and approved the final version of the manuscript.

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Abbreviations

NSCLC non-small cell lung cancer
DHA dihydroartemisinin.

Conflicts of interest**:** *Declared none.*

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