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The inhibitory effect of dihydroartemisinin on the growth of neuroblastoma cells

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EDITOR'S NOTE

Nobel prize winner Tu Youyou, with her academic team found artemisinin (qinghaosu) and dihydroartemisinin (dihydroqinghaosu). "Her work laid the most important foundation for the treatment of malaria by using artemisinin, got vigorous promotion by China and World Health Organization, saved millions of lives of patients suffered from malaria world-wide, especially those from developing countries, and made an outstanding contribution to the treatment and control of this important parasitic disease." [1]. In recent years, some scientists reported that dihydroartemisinin also has another advantage of killing multiple cancer cells. The results of the present study showed that dihydroartemisinin could inhibit the proliferation of neuroblastoma cells SH-SY5Y.

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ABSTRACT

Objective: To evaluate the inhibitory effect of dihydroartemisinin on neuroblastoma cell line SH-SY5Y, explore the possible mechanism of dihydroartemisinin against neuroblastoma cells.

Methods: The cell viability of dihydroartemisinin treated SH-SY5Y cells was examined by MTT assay and morphology of cells was observed by using inverted microscope. Cell cycle was examined with flowcytometry assay, then cyclin D1 and caspase-3 proteins expression was detected by ELISA and western blotting assay.

Results: MTT analysis results showed that cell viability significantly decreased after exposure to 0.05, 0.50, 5.00 and 50.00 μmol/L dihydroartemisinin in a dose-dependent manner, and the lower density of cells was observed in treated groups. The number of cells in sub-G1 phase was increased after treatment with different doses of dihydroartemisinin compared with the control group. The expression of cyclin D1 protein was decreased, while the expression of caspase-3 protein was increased in treated group.

Conclusions: Dihydroartemisinin could inhibit the proliferation through stopping the cell cycle and inducing the apoptosis in neuroblastoma SH-SY5Y cells.

1. Introduction

Neuroblastoma is one of the most common malignant solid tumors in infants and young children [2]. So far, there has been no effective treatment. Surgery, chemotherapy and radiotherapy are the three main methods clinically. Among them, chemotherapy drugs could kill the tumor cells, but at the same time, they can also bring huge toxic side effects, and lead to serious impact on physical and mental health of children. Therefore, it is necessary to find a kind of chemotherapeutic drugs with low toxicity and high efficiency for neuroblastoma treatments.

Dihydroartemisinin (DHA), a main active metabolite extracted from artemisinin, has been widely used as an antimalaria drug clinically. It possesses many advantages such as good absorption, wide distribution, rapid excretion and metabolism, high efficiency and low toxicity, *etc*. In the study by Hou *et al.*, the result showed that DHA has less effects on the growth of normal cells [3], but it could significantly kill multiple cancer cells *in vivo* [4].

The present study aimed to explore the inhibition on neuroblastoma SH-SY5Y cells proliferation by DHA using MTT assay and morphology detection, thereby investigating the possible mechanisms responsible for DHA-induced inhibition

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on SH-SY5Y cells by flow cytometry, ELISA and western blotting assay.

2. Materials and methods

2.1. Drug and reagents

In this study, DHA was purchased from Chengdu Okay Co., Ltd. Dulbecco's modified Eagle medium was from Hyclone Co. (Logan, Utah, USA). Trypsin (1:250) and fetal bovine serum were from Invitrogen Co. (Carlsbad, CA, USA). Dimethylsulfoxide (DMSO) and MTT used in the experiment were from Sigma Chemical Co. (St. Louis, MO, USA). Cyclin D1, caspase-3 and β-actin antibodies were purchased from Santa Cruz Co. DHA was dissolved in DMSO solution at 50 mmol/L and reserved in refrigerator.

2.2. Cell line and cell culture

The human neuroblastoma cell line SH-SY5Y was provided by Scientific Research Center of Jinlin Medical University. The experiments using human cell lines were approved by Jinlin Medical University Ethics Committee. The cryopreserved SH-SY5Y cells were placed in a 37 °C water bath and vibrated until dissolved. After centrifugalization at 1 000 r/min for 5 min, the cell suspension was then cultured in the Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum at 37 °C, in a humidified atmosphere of 95% O_2 and 5% CO_2. The cells were subcultured every 2 or 3 days.

2.3. Cell viability assay

Cells were cultured in 96-well plate at a density of 8×10^3 cells/well and incubated for 24 h. Various doses of DHA (0.00, 0.05, 0.50, 5.00 and 50.00 μmol/L) were used to treat the cells for 24, 48 and 72 h. After incubation, 20 μL MTT (5 mg/mL) reagent was added to each well for 4 h at 37 °C. Then the MTT liquid was replaced, and 150 μL DMSO was added and the mixture was agitated for 10 min. Absorbance was read at a wavelength of 490 nm by using an EL×800 Universal Microplate Reader (BIO-TEK, Norcross, GA, USA). Experiments were performed in triplicate, and cell viability was calculated as a percentage of the control (the group treated with 0.00 μmol/L DHA).

2.4. The morphology of SH-SY5Y cells

Cells were cultured overnight in 25 cm^2 flask, and then treated with 0.05, 0.50, 5.00 and 50.00 μmol/L DHA for 24 h, respectively. Cells treated with vehicle served as control. Photos were taken under inverted microscope (Olympus Corporation, Japan).

2.5. Cell cycle assay

Cells were collected after 24 h treatment with DHA at 0.05, 0.50, 5.00 and 50.00 μmol/L, respectively, and washed with phosphate-buffered saline solution, then resuspended in 80% ice-cold methanol and incubated at −20 °C overnight. Cells were stained with buffered saline solution containing 20 μg/mL propidium iodide for 30 min, filtered with 300-mesh nylon screen, and then analyzed with flow cytometry (Epics-XL, Beckman Coulter Inc., Bria, CA, USA).

2.6. ELISA assay

The SH-SY5Y cells were divided into 5 groups, and treated with 0.00, 0.05, 0.50, 5.00 and 50.00 μmol/L DHA for 24 h, respectively. The supernatant was collected for experiments, and then incubated with coating buffer at ratio of 1:1 overnight at 4 °C. After closure, the cyclin D1 and caspase-3 antibodies (1:1 000 dilution) were separately added, followed by continued incubation overnight at 4 °C. Then the cells were incubated at 37 °C for 1.5 h after adding secondary antibody (1:1 000 dilution). Diaminobenzidine was added for 5 min; when the color was brown, the absorbance value was determined by automatic microplate reader (MDC, USA) at 492 nm.

2.7. Western blotting

SH-SY5Y cells were collected after 0.05, 0.50, 5.00 and 50.00 μmol/L DHA treatments, and lysed with a lysis buffer (50 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L ethylene diamine tetraacetic acid, 10% glycerol, 1% Triton X-100, 1% protease inhibitor mixture and 1 mmol/L phenylmethanesulfonyl fluoride). Fifty micrograms of total protein from each lysate were separated through SDS-PAGE and transferred to membranes. The membranes were incubated with the primary antibodies against β-actin, cyclin D1 and caspase-3. β-actin, as a loading control (1:1 000; SANTA CRUZ, USA), was used overnight at 4 °C. Primary antibody binding was detected with anti-rabbit immunoglobulin G conjugated to horseradish peroxidase and visualized by an enhanced chemiluminescence detection system (Pierce, Rockford, IL, USA). Results were analyzed using Image-Pro Plus image analysis and management systems (USA).

2.8. Statistical analysis

Data were analyzed by the SPSS 17.0 software and expressed as mean ± SD. Statistical comparisons between different groups were done by using One-way ANOVA. The statistical significance was determined at $P < 0.05$.

3. Results

3.1. The effects of DHA on the proliferation of SH-SY5Y cells

The results of MTT assay showed that different concentrations of DHA could inhibit the growth of SH-SY5Y cells at 24, 48 and 72 h, respectively; the proliferation was significantly decreased in 0.50, 5.00 and 50.00 μmol/L DHA treated groups ($P < 0.05$, $P < 0.01$). DHA could inhibit the growth of SH-SY5Y cells in a dose-dependent manner. The inhibition was the most obvious at 24 h, therefore 24 h was taken as the proper time for the follow-up experiments (Table 1).

The morphology of cells was observed under the inverted microscope. The number of adherent cells in DHA treated groups was significantly decreased compared with the control group, and at the same time, the density of cells was decreased with increasing concentrations of DHA (Figure 1).

Table 1
Effects of DHA on the growth of SH-SY5Y cells. %.

Groups	The rates of proliferation		
	24 h	48 h	72 h
Control group	100.00 ± 1.36	100.00 ± 4.54	100.00 ± 4.61
0.05 μmol/L DHA group	94.52 ± 1.45	98.49 ± 1.53	90.76 ± 3.38
0.50 μmol/L DHA group	93.15 ± 4.41*	95.46 ± 1.58*	89.23 ± 3.44*
5.00 μmol/L DHA group	86.30 ± 1.58**	92.43 ± 4.92**	83.07 ± 3.70**
50.00 μmol/L DHA group	79.45 ± 3.45**	84.85 ± 3.57**	84.61 ± 1.81**

*: $P < 0.05$ compared with control group. **: $P < 0.01$ compared with control group. Data were expressed as mean ± SD.

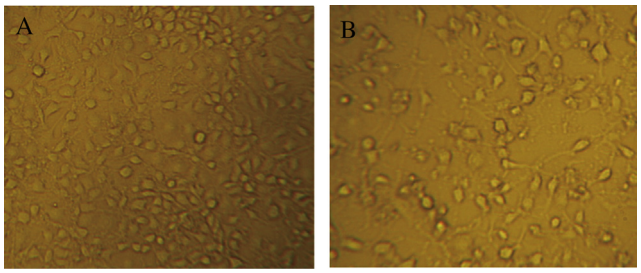

Figure 1. DHA inhibited the growth of SH-SY5Y cells (×200).
A: Control group; B: Cells treated with 50 μmol/L DHA.

3.2. The changes of cell cycle after DHA treatments

The number of SH-SY5Y cells in sub-G1 phase was increased with the increase in concentrations of DHA. In this phase, compared with the control group, the cells of 50 μmol/L DHA group were significantly increased ($P < 0.05$). The number of SH-SY5Y cells in G0/G1 phase was increased at first and then decreased. The number of SH-SY5Y cells in S and G2/M phases was decreased (Table 2).

Table 3
The secretion of cyclin D1 and caspase-3 proteins in supernatant.

Groups	Absorbance value	
	Cyclin D1	Caspase-3
Control group	0.90 ± 0.03	1.05 ± 0.04
0.05 μmol/L DHA group	0.85 ± 0.12	1.06 ± 0.03
0.50 μmol/L DHA group	0.83 ± 0.04	1.07 ± 0.02
5.00 μmol/L DHA group	0.82 ± 0.01	1.08 ± 0.01
50.00 μmol/L DHA group	0.77 ± 0.07*	1.09 ± 0.02

*: $P < 0.05$ compared with control group. Data were expressed as mean ± SD.

The results of western blotting showed that the expression of cyclin D1 was decreased in DHA treated groups, and the difference was statistically significant ($P < 0.05$, $P < 0.01$), while caspase-3 protein was increased in different DHA doses treated groups when compared with control group, and the difference between 50 μmol/L DHA group and control group was statistically significant ($P < 0.01$) (Figure 2).

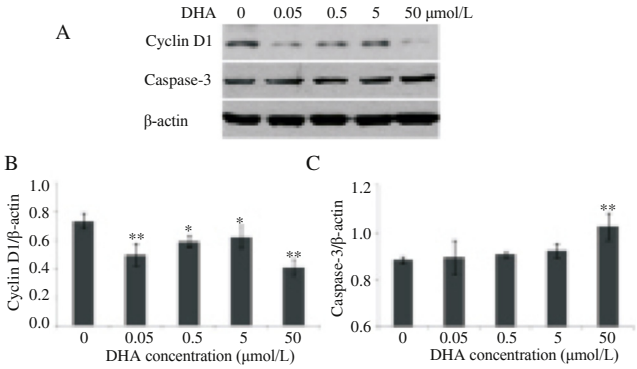

Figure 2. DHA affected the expression of cyclin D1 and caspase-3 proteins in SH-SY5Y cells.
A: The expression of cyclinD1 and caspase-3 proteins; B: The level of cyclin D1 protein expressed; C: The level of caspase-3 protein expressed. *: $P < 0.05$ compared with control group; **: $P < 0.01$ compared with control group. Data were expressed as mean ± SD.

Table 2
Effect of DHA treatment on cell cycle of SH-SY5Y cells. %.

Groups	Cell cycle			
	Sub-G1	G0/G1	S	G2/M
Control group	2.08 ± 0.28	53.78 ± 1.23	23.17 ± 1.17	20.97 ± 1.03
0.05 μmol/L DHA group	4.98 ± 0.29	56.05 ± 1.32	23.02 ± 1.25	15.95 ± 1.21
0.50 μmol/L DHA group	8.46 ± 0.53	55.94 ± 2.11	22.74 ± 1.38	12.86 ± 0.98
5.00 μmol/L DHA group	10.11 ± 0.81	55.24 ± 3.35	22.36 ± 2.02	12.29 ± 0.85
50.00 μmol/L DHA group	15.28 ± 1.19*	53.26 ± 2.28	19.42 ± 1.01	11.94 ± 1.04

*: $P < 0.05$ compared with control group. Data were expressed as mean ± SD.

3.3. The expression of cyclin D1 and caspase-3 proteins

The results of ELISA showed that the secretion of cyclin D1 protein was decreased in cultured supernatant; when compared with control group, the secretion of cyclin D1 in 50 μmol/L DHA group was significantly reduced ($P < 0.05$). However, the secretion of caspase-3 protein was increased in all DHA treated groups compared with the control group without significant difference ($P > 0.05$) (Table 3).

4. Discussion

Artemisinin is a kind of compound which contains the peroxide groups and is extracted from *Artemisia annua* L. It is an effective and famous natural product for clinical treatment of malaria, otherwise as a new anti-tumor agent [5]. DHA is the main active metabolite of artemisinin *in vivo*, whose oral bioavailability is 10 times higher than that of artemisinin, and the anti-malaria effects are stronger than artemisinin by 4–8

times; at the same time, DHA also has an obvious anti-tumor activity [5–8]. However, the inhibitory effect of DHA on the growth of SH-SY5Y cells is still unknown.

We studied how various doses of DHA affected the proliferation of SH-SY5Y cells, and thereby explored its anti-tumor function. The results revealed that different concentrations of DHA can significantly inhibit the growth of neuroblastoma cells at indicated time points, and the inhibitory effect was obviously dose-dependent. The density of cells was obviously decreased with increased doses of DHA. These results showed that DHA inhibited the growth of SH-SY5Y cells in a concentration dependent manner.

The results regarding cell cycle showed that the number of SH-SY5Y cells was increased in sub-G1 phase, increased and then decreased in G0/G1 phase, decreased in S and G2/M phases with the increased concentrations of DHA. As it is known to all, the growth of the cells is regulated by cyclins and cyclin-dependent kinase inhibitor, among which cyclin D1 plays a positive regulatory role in the growth of cells [8]. Hosoya *et al.* and Mao *et al.* reported that DHA stopped the cell cycle at G0/G1 phase, and made a problem in the conversion of G1/S junction [9,10]. Cyclin D1 is an important protein which regulates the conversion of G1/S junction; blocking and knocking out cyclin D1 could stop the cell cycle at G0/G1 phase, and induce apoptosis [11]. Therefore, our results suggested that DHA could down-regulate the expression and secretion of cyclin D1 protein to stop cell cycle and inhibit the proliferation of SH-SY5Y cells.

Apoptosis is a process of programmed cell death controlled by multiple factors, which is caused by a series of intracellular proteins. The caspases play an important role in the process of cell apoptosis [12]. Although caspase is rarely expressed in most tumor tissues [13], it is the only way for the caspases cascade reaction, and it is also the key molecule in apoptosis. Casepase-3 is an important protein which exists in the form of proenzyme in cytoplasm; it is activated when apoptosis is induced in tumor cells [14]. DHA induced the apoptosis through caspases pathway [15]. Our results showed that there was an increase in the secretion and expression of caspase-3 protein by SH-SY5Y cells, which certified that DHA could induce apoptosis through caspase-3 activation.

In conclusion, DHA could induce apoptosis of neuroblastoma SH-SY5Y cells through down-regulating cylin D1 and activating caspase-3, thereby inhibiting the growth of neuroblastoma cell. However, because there are many factors affecting the growth of tumor cell, and among them, the cell cycle and apoptosis were preliminarily studied in the present study, further investigation of the mechanism of DHA against tumor cells is still needed in the future study.

Conflict of interest statement

We declare that we have no conflict of interest.

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