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Effect of combined treatment with cyclophosphamidum and allicin on neuroblastoma-bearing mice

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ABSTRACT

Objective: To evaluate the efficacy of allicin combined with cyclophosphamide on neuroblastoma (NB)-bearing mice and explore the immunological mechanism in it. **Methods:** A total of 30 NB-bearing mice were equally randomized into model group, cyclophosphamide group and combined therapy group, 10 nudemice were set as normal saline (NS) group. Cyclophosphamide group and combined therapy group were weekly injected with 60 mg/kg cyclophosphamide for four weeks; besides, combined therapy group was given with allicin (10 mg/kg/d) by gastric perfusion for 4 weeks; model group and NS group were given with the same volume of NS. Serum VEGF content was detected by ELISA pre-treating (0 d) and on the 3rd d, 14th d and 28th d; on 29th d, all mice were sacrificed and the tumor, liver, spleen and thymic tissues were weighted. Tumors were made into paraffin section for detecting tumor cell apoptosis and proliferation by TUNEL and BrdU method, respectively. Survival curves were drawn by Kaplan-Meier method. **Results:** After treatment, both treatment groups relieved on viscera indexes, VEGF level, T cell subsets distribution and tumor growth and each index of combined therapy group was better than cyclophosphamide group (P<0.05 or 0.01); only combined therapy group could significantly increase the lifetime of NB-bearing mice (χ^2=5.667, P=0.017). **Conclusions:** Allicin can improve T cell subsets distribution and inhibit VEGF expression through its immunomodulatory activity, thereby improve the efficiency on NB in coordination with cyclophosphamide.

1. Introduction

Neuroblastoma (NB) is the most common and lethal extracranial tumor in children. It accounted for 8%-10% of cancer incidence and 12% of cancer mortality in children[1]. The five-year survival rate for children with high risk NB was only 30%-50%[2]. Although the intensive chemotherapy, radiation, hematopoietic stem cell transplantation technology had been made considerable progress, the prognosis of refractory NB was poor[3]. In clinic, variations of karyotype and cell characteristics, such as tumor suppressor genes mutations, chromosome recombination or deficiency, usually caused tumor to evade treatments of most drugs or drug resistance[4]. Therefore, searching for novel therapeutic compounds or therapeutic measures that can work on a wide range of NB cells is desperately needed for NB therapy[5].

Allicin is the major component and the most biological compound of pulverized fresh garlic[6-8]. Allicin has obvious inhibitory effects on different kinds of tumor cells such as gastric cancer, colon cancer, liver cancer, and lung cancer and has been used for clinical therapy as an aid cancer drug. In the study, we established NB-bearing mice model and treated them with different schemes to explore the feasibility and efficacy of combined treatment with cyclophosphamidum and allicin on NB.

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2. Material and methods

2.1. Experimental animals and cell lines

A total of 50 four-week-old female nudemice (BALB/c-nu/nu) weighing 12-14 g were bought from Shanghai Laboratory Animal Centre of Chinese Academy of Sciences. The animals were maintained at 25 ℃ with a 12-h light/12-h dark cycle in laminar flow cabinets under specific pathogen-free conditions and given with sterile water and food *ad libitum*.

The cell line used in experiment was human neuroblastoma SH-SY5Y cell line bought from Tumor Cell Bank of Chinese Academy of Medical Sciences.

2.2. Establishment of tumor-bearing mice model and grouping

When SH-SY5Y cell morphology was well, mice would be injected. The skin of right forelimbs flank were disinfected, then cell suspension at 2×10^7-3×10^7 cells/mL was injected (0.1 mL for one mouse). A total of 40 nudemice were injected, the remaining 10 nudemice were injected with 0.1 mL normal saline (NS) and set as NS group.

About 7-9 d after injecting cell suspension (at this time the tumor volume in mice was about 100 mm^3), 30 mice were modeled successfully. They were equally randomized into three groups: model group, cyclophosphamide group and combined therapy group. The day of grouping was regarded as the 1st d of treatment (d1). On the 1st d, cyclophosphamide group and combined therapy group were injected with cyclophosphamidum 60 mg/kg through caudal vein for the first time and on the 8th d for the second time and so on, a total of four times. Besides, combined therapy group was given allicin (10 mg/kg/d) by gastric perfusion for four weeks. Model group and NS group were given the same volume of NS.

After four-week treatment, on 29th d, all mice were sacrificed and their tumor, liver, spleen and thymic tissues were collected for subsequent tests.

2.3. Detection of serum vascular endothelial growth factor (VEGF)

In order to monitor the curative effect of two treatment groups, pre-treating (0 d) and on the 3rd d, 14th d, 28th d of the treatment, peripheral blood of each group was collected through its submandibular vein and centrifuged at 3 000 rpm for 10 min, and the supernatant was reserved. After collecting all samples, the content of VEGF protein in serum was detected by using Mouse VEGF ELISA kit.

2.4. Determining of T lymphocyte subsets in peripheral blood

The detection time points of T lymphocyte subsets in blood samples were same to those in 2.3. The distribution of T lymphocyte subsets ($CD3^+$, $CD4^+$, $CD8^+$) was analyzed by flow cytometry.

2.5. Histology and immunostaining

Formalin-fixed paraffin-embedded sections from model group and two treatment groups were stained with H&E or subjected to immunohistochemistry (IHC) with specific antibodies. Three hours before sacrifice, mice were injected with BrdU (1 mL/100 g body weight) to evaluate the proliferation rate of tumor cells[9]. Tumor cells apoptosis rate was detected by TUNEL method. Apoptotic index (AI) = apoptosis cell numbers/(apoptosis cell numbers+non-apoptosis cell numbers)×100%. All immunostains were evaluated in 10 random microscopic fields selected in viable tumor regions only (magnification×200).

2.6. Data processing and analysis

All data was processed by SPSS16.0 software. Data was presented as mean±standard deviation of at least three independent experiments. Comparisons were made among the groups using One-way ANOVA followed by Tukey-Kramer test or Bonferroni test. Survival curves were drawn by Kaplan-Meier method and overall comparison and pairwise comparisons were conducted by Log-Rank method. A P-value <0.05 was considered significantly different.

3. Results

3.1. Curative effect in each group

After four-week treatment, the tumor weight and visceral indexes of cyclophosphamide group and combined therapy group both relieved more significantly than those in model group (P<0.05 or 0.01), and each index of combined therapy group was better than cyclophosphamide group (P<0.05 or 0.01), especially the tumor weight and spleen index (P<0.01) (Table 1). Figure 1 showed that compared with model group, both treatment groups could improve the proportions of $CD3^+$, $CD4^+$ T lymphocytes (P<0.05 or 0.01), and combined therapy group could also significantly reduce the proportion of $CD8^+$ T lymphocytes (P<0.05). On the 3rd d of treatment, $CD4^+/CD8^+$ ratio in combined therapy group was higher than that in model group (P<0.05); on the 14th d, $CD4^+/CD8^+$ ratio of cyclophosphamide group began to be higher than that in model group (P<0.05). Overall, combined therapy could more timely improve the distribution of T cell subsets than cyclophosphamide monotherapy.

Before treatment, the serum VEGF content of the three NB-bearing mice groups were similar; after starting treatment,

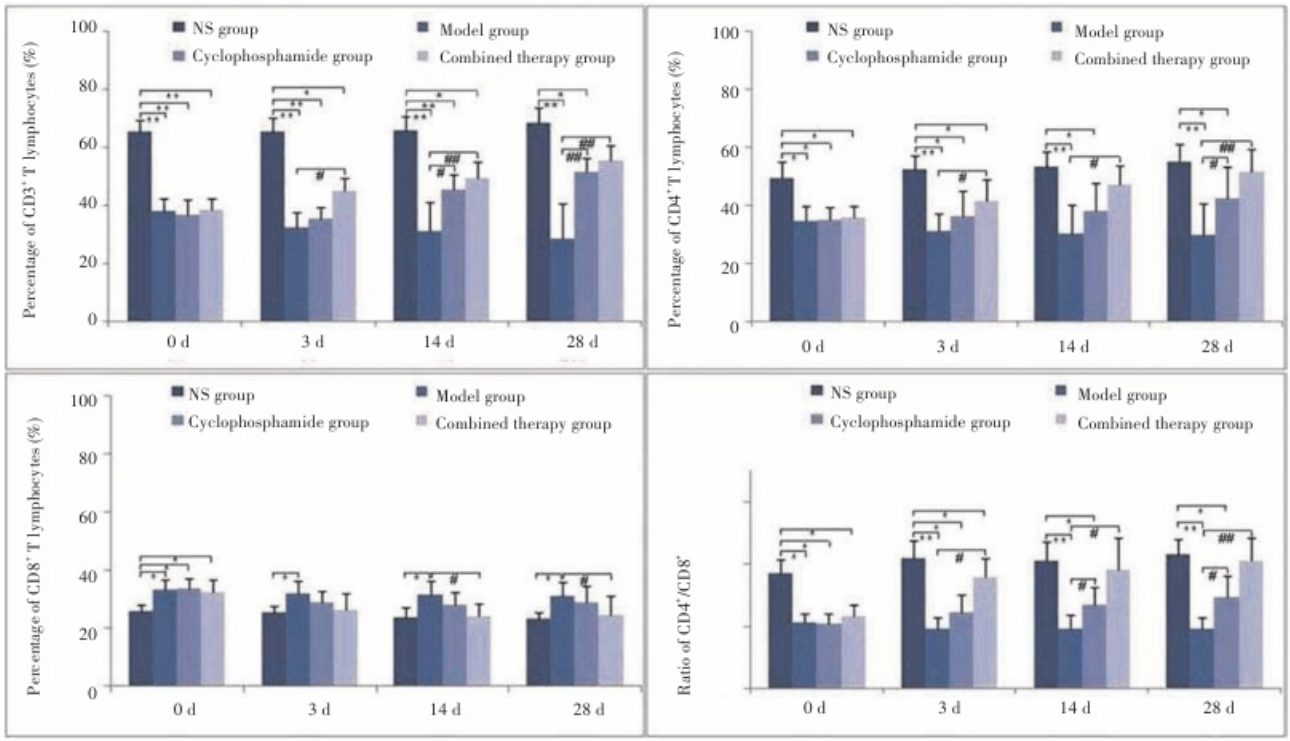

Figure 1. Proportion of T lymphocyte subsets in peripheral blood at different time points.
Note: * *vs.* NS group, $P<0.05$; ** *vs.* NS group, $P<0.01$; # *vs.* Model group, $P<0.05$; ## *vs.* Model group, $P<0.01$.

VEGF content in both treatment groups decreased rapidly ($P<0.05$ or 0.01); once 4-week treatment finished, VEGF content in combined therapy group and in NS group was undifferentiated ($P>0.05$) (Figure 2).

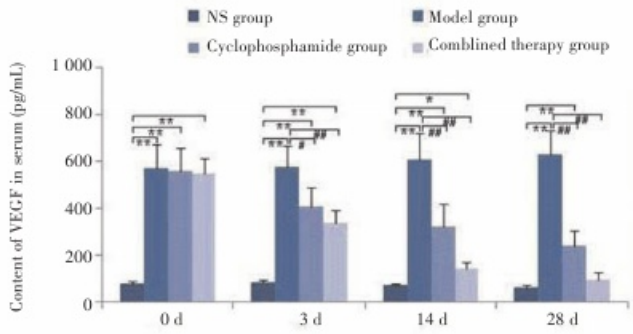

Figure 2. Serum VEGF content of mice in each group before and after therapy.
Note: * *vs.* NS group, $P<0.05$; ** *vs.* NS group, $P<0.01$; # *vs.* Model group, $P<0.05$; ## *vs.* Model group, $P<0.01$.

3.2. Survival analysis of animals in each group

Figure 3 showed that the survival curves of mice in different group compared by Log-Rank method were statistically significant($\chi^2=13.387$, $P=0.004$). By the end of treatment, the survival rates of two treatment groups were higher than model group, and the survival rate of combined therapy group was also higher than cyclophosphamide group. Compared with model group, combined therapy group could significantly prolong the lifetime of tumor-bearing mice($\chi^2=5.667$, $P=0.017$),while cyclophosphamide monotherapy could not reach this effect($\chi^2=1.570$, $P=0.210$).

3.3. Analysis of tumor cells proliferation and apoptosis

Figure 4 and Figure 5 showed that the tumor cells proliferation rate in combined therapy group and cyclophosphamide group both decreased while the apoptosis rate in both groups increased ($P<0.05$ or 0.01); proliferation inhibition effect and apoptosis promoting effect in combined therapy group were more obvious than those in

Table 1
Tumor-inhibition rate and visceral indexes in each group.

Groups	Tumor weight (g)	Tumor-inhibition rate (%)	Liver index (mg/g)	Spleen index (mg/g)	Thymus index (mg/g)
NS group	-	-	4.78±0.42	7.11±0.33	3.20±1.11
Model group	0.72±0.48	-	$7.12\pm0.93^{*}$	$1.60\pm0.22^{**}$	$1.08\pm0.26^{**}$
Cyclophosphamide group	$0.17\pm0.10^{\#\#}$	63.1	$6.52\pm0.71^{*}$	$2.73\pm0.98^{**\#}$	$2.10\pm0.84^{**\#\#}$
Combined therapy group	$0.05\pm0.03^{\#\#\triangledown\triangledown}$	92.6	$5.10\pm0.55^{\#\triangledown}$	$5.49\pm1.10^{*\#\#\triangledown\triangledown}$	$2.95\pm1.05^{\#\#\triangledown}$

Note: * *vs.* NS group, $P<0.05$; ** *vs.* NS group, $P<0.01$; # *vs.* Model group, $P<0.05$; ## *vs.* Model group, $P<0.01$; $\triangledown$ *vs.* Cyclophosphamide group, $P<0.05$; $\triangledown\triangledown$ *vs.* Cyclophosphamide group, $P<0.01$.

cyclophosphamide group (P<0.05 or 0.01).

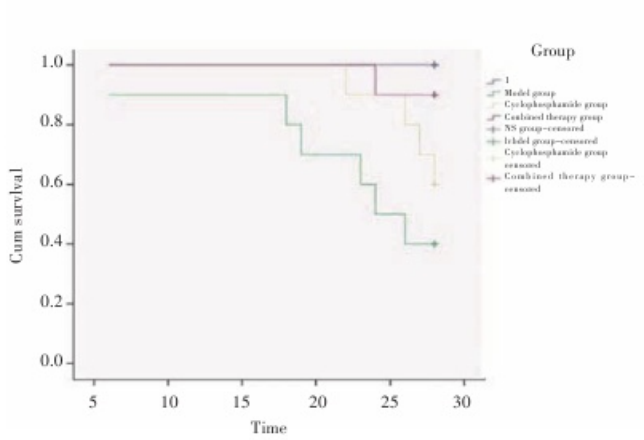

Figure 3. Survival curves of mice in each group.

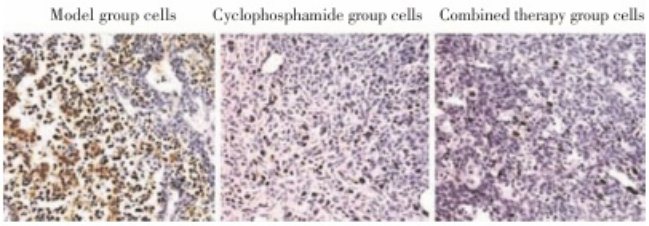

Figure 4. Determining of tumor cells proliferation rate in three NB-bearing mice groups by BrdU method.

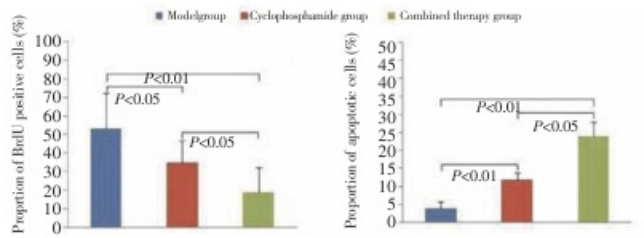

Figure 5. Comparisons of tumor cells proliferation and apoptosis in three NB-bearing mice groups.

4. Discussion

Allicin is a sulfur compound extracted from garlic bulbs[10]. The surveys of epidemiology showed allicin had inhibition effect on many malignant tumors[11-13]. In the study, we combined the treatment with cyclophosphamidum and allicin on NB-bearing mice for the first time. Our results displayed that compared with cyclophosphamide monotherapy, the combined therapy could better inhibit the tumor growth in NB-bearing mice and improve T lymphocyte subsets distribution and tumor-bearing mice lifetime (P<0.05 or 0.01). Besides, we detected VEGF, the molecular marker of NB, and found after the combined therapy the serum VEGF content of NB-bearing mice was same to normal nudemice (P>0.05).

These above results suggested that allicin had obvious inhibition effect on NB and could increase curative effect of cyclophosphamidum. In fact, allicin used for fighting tumor had been reported many times, and the molecular mechanism of its anti-tumor effect often was attributed to tumor cells apoptosis induced by allicin[14]. It had become clear that the induction of apoptosis was crucial for the anti-cancer effect of allicin. In yeast system,allicin also caused a redox-shift in human cell cultures[15] which led to the execution of tumor cells death which depended on caspases or was independent of caspases[16,17].

Tao *et al* studied the proliferation inhibition effect of allicin on gastric cancer cell line SGC-7901, they found allicin could significantly inhibit the proliferation of this cell line and induce the apoptosis, with its proliferation inhibition effect on cancer cell in concentration-dependent manner[18]. Wang *et al* applied the combined treatment with recombined human IL-2 and allicin to treat the pancreatic tumor-bearing mice, and their results also showed after the combined therapy the survival time of tumor-bearing mice was obviously prolonged, the tumor growth was inhibited, and the serum $CD4^+$ cells, $CD8^+$ cells, NK cells and IFN-γ level had been more significantly improved than monotherapy group. They thought that this anti-tumor effect was achieved by activating $CD4^+$ cells, $CD8^+$ cells and NK cells[19]. Jiang *et al*[20] certified that the combined therapy of artesunate and allicin could inhibit obviously the viability of osterosarcoma cells in a concentration and time dependent manner; and moreover, invasion, motility and colony formation ability were significantly suppressed through caspase-3/9 expression and activity enhancement. Yang RK and his colleagues utilized a mouse model to investigate the impact of tumor burden on hu14.18-IL-2 treatment efficacy in IV- versus IT-treated animals, and their results showed that smaller tumor burden at treatment initiation was associated with increased infiltration of NK and $CD8^+$ T cells and increased overall survival[21]. Furthermore, allicin acted on T-cell lymphocytes by inhibition of the SDF1 α -chemokine-induced chemotaxis and this effect was correlated with an impaired dynamic of the actin-cytoskeleton[22]. Before treatment, the $CD3^+$ cell level and $CD4^+/CD8^+$ ratio in tumor-bearing mice were lower than normal level, which suggested that the immune system was compromised and in immunosuppression state. After treatment (especially the combined treatment), these indexes were improved significantly which may be caused by immune modulating activity of allicin[14,23]. Besides, after treatment, the serum VEGF content of NB-bearing mice decreased and even equal to the level of mice in NS group, which maybe caused by allicin directly inhibiting the expression of VEGF mRNA[24].

When we design the experiment, the NB-bearing mice were given allicin (10 mg/kd/d) by gavage to strengthen

the anti-tumor effect of cyclophosphamidum. While *in vivo* experiment, it is still unknown thatwhether allicin strengthened anti-tumor effect of cyclophosphamidum in a concentration or time dependent manner. Therefore, the further research needs to confirm the manner of allicin exerting its anti-tumor effect.

In conclusion, allicin had unique anti-tumor effect on the treatment of NB and could produce synergetic effect combining with cyclophosphamide. Allicin exerted its curative effect through improving T cell subsets distribution and cellular immunity.

Conflict of interest statement

We declare that we have no conflict of interest.

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