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Study on the influence of curcumin on chemosensitivity of nephroblastoma cells

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ABSTRACT

Objective: To study the influence of curcumin on chemosensitivity of nephroblastoma cells.**Methods:** Human nephroblastoma cells line SK-NEP-1 was transplanted to the nude mice subcutaneously to establish the implantation tumor model of human nephroblastoma cells. A total of 30 tumor-bearing mice were divided into three groups of ten randomly. The routine chemotherapy group was given vincristine (0.05 mg/mL·0.2 mL/d) and actinomycin D (15 ng/mL·0.2 mL/d) combined chemotherapy regime. The curcumin chemotherapy group was given the same combined chemotherapy regimens and curcumin (30 mg/kg/d) by intraperitoneal injection. The control group was given normal saline (NS) of the same volume by intraperitoneal injection. Continuous administration would be kept for 4 weeks and 3 days a week. The volumetric changes of every group were recorded. The serum of every group in different time was collected and the VEGF content was detected by ELISA. All mice were sacrificed and the tumor tissues were stripped and weighed after 4 weeks' treatment. The tumor inhibition rate was calculated. The cell proliferation activity and apoptosis rate were detected by MTT and flow cytometry method. All data were statistically analyzed by SPSS 19.0.**Results:** The tumor volume, serum VEGF content, tumor inhibition rate, cell proliferation activity and apoptosis rate of routine chemotherapy group and curcumin chemotherapy group had significant differences comparing with the control group ($P < 0.05$) after 4-week's treatment. The cancer growth of curcumin chemotherapy group was obviously decreased and even tended to shrink comparing with routine chemotherapy group ($\chi^2 = 15.732, P = 0.007$). The cell proliferation activity was significantly reduced and the apoptosis rate was significantly higher, ($\chi^2 = 9.427, P = 0.012$) which showing the effect of chemotherapy was enhanced.**Conclusions:** The chemosensitivity of nephroblastoma cells could be improved by curcumin, then the effect of preoperative adjuvant chemotherapy scheme would be enhanced, the growth of nephroblastoma cells would be inhibited and the surgical risk of nephroblastoma would be reduced.

1. Introduction

Nephroblastoma is one of the most common malignant tumors in children. The incidence of this tumor is only second to

neuroblastoma. Pathogenesis is generally considered to be chromosomal deletions or familial inheritance [1], resulting in the differentiation disorder and malignant proliferation of blastemal cells. Thus, the age of onset is generally less than 5 years old, a few are less than 10 years old [2]. In the early stage of the disease, the survival rate is low due to the overall backwardness of tumor clinical research. In recent years, compared with the cure rate of malignant tumors in adults, related researches have improved the cure rate of this disease significantly. Modern clinical treatment measures are

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radiochemotherapy and surgical comprehensive treatment. Five-year's survival rate is generally above 80%. Studies have shown that preoperative adjuvant chemotherapy could effectively reduce the surgical risk of nephroblastoma and improve the prognostic effects [3]. Therefore, preoperative chemotherapy has a pivotal position in the whole process of treatment. Currently, limited drugs used for children's tumor chemotherapy are mainly vincristine, actinomycin D, adriamycin Amycin *etc.* [4,5]. Chemotherapy adapts one or more drugs according to tumor stage, and the effect are different because of children's individual factors. Therefore, the study of nephroblastoma aims to explore a new and effective adjuvant chemotherapy scheme, and improve the effect of preoperative chemotherapy and chemosensitivity.

Curcumin, extracted from curcuma, is an important component of Chinese medicine with the effect of softening heart and cerebral vessels, antioxidation, antiseptis, anti-inflammation and regulating the digestive system [6]. Studies have shown that this compound can inhibit the malignant proliferation of tumor cells [7,8], and enhance chemosensitivity of liver cancer, gastric cancer and colon cancer *etc.* [9,10], but the research applied to pediatric tumor is rare. This study provides theoretical and experimental basis for the study of new adjuvant chemotherapy, by establishing the implantation tumor model of nephroblastoma cells and implementing different chemotherapy schemes to research the influence of curcumin on chemosensitivity of nephroblastoma cells.

2. Materials and methods

2.1. Materials

Nude mice used in the experiment were purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences. A total of 36 mice which were 4–5 weeks old were used for this study. Half of them were male mice and half were female mice. The study was performed after the mice were allowed to acclimate for 1 week at Animal Experimental Center of Zhengzhou University. Laboratory was kept sterilized with constant temperature and humidity.

Human nephroblastoma cell line SK-NEP-1 was purchased from the Shanghai Cell Bank, Chinese Academy of Sciences. Curcumin was purchased from Sigma Company. RPMI-1640 and fetal bovine serum were purchased from GIBCO Company. Rat VEGF ELISA kit was purchased from Santa Cruz Company; DMSO and MTT were purchased from Sigma Company. FITC, and PI were purchased from BD (Shanghai) Co., Ltd. Among them, curcumin was dissolved in 10% DMSO-normal saline before 30 mg/kg clear liquid was formulated.

2.2. Methods

2.2.1. Implantation tumor model

The cell cryopreserved tubes were placed in the 37 °C water bath and shaken for thawing out. The cell suspension was transferred to culture bottles with suction tubes and then cultured in RPMI-1640 medium after adding 10% fetal bovine serum and

100 U/mL penicillin and streptomycin. Cells were digested by trypsin every other day and the culture medium was replaced to log phase.

The cell concentration was adjusted to 1×10^7 /mL. Before the experiment, right forelimbs of mice were disinfected by iodine, and injected with 0.1 mL cell suspension in 1 mL sterile syringes. After implantation, mice were under close observation for 30 min, and were put back to the cage without adverse reaction.

2.2.2. Treatment in each group

After 7–10 days, 30 mice implanted tumor models were divided into 3 groups. They are routine chemotherapy group, curcumin chemotherapy group and control group respectively. The routine chemotherapy group was intraperitoneally injected with vincristine (0.05 mg/mL·0.2 mL/d) and actinomycin D (15 ng/mL·0.2 mL/d). Continuous administration was 3 days a week and kept for 4 weeks. The curcumin chemotherapy group was given the same combined chemotherapy regimens and curcumin (30 mg/kg/d) by intraperitoneal injection. Continuous administration was 3 days a week and kept for 4 weeks. The control group was given normal saline (NS) of the same volume by intraperitoneal injection. After treatment, tumor's long (a) and short diameters (b) were measured every 3 days, and the tumor volume was calculated with the formula $V = 0.5 \times ab^2$.

2.2.3. Detection of the VEGF content

two mice were randomly selected 1 day before treatment and 7 d, 14 d, 21 d and 28 d after treatment, and 2 mL peripheral blood were drew by femoral artery blood sampling method. The serum was obtained after 3 000 rpm centrifugation, and the VEGF protein content in serum samples was detected by ELISA. The specific operating steps were carried out according to the instructions of kit.

2.2.4. Cells proliferation activity

After 4 weeks' chemotherapy, all mice were sacrificed with cervical dislocation and the tumor tissues were stripped and weighed immediately. The tumor inhibition rate was calculated as:

Tumor inhibition rate = (tumor weight of control group – tumor weight of experimental group)/tumor weight of control group $\times 100\%$.

Cells suspension was made from tumor specimens and cultured in DMEM medium containing 20% fetal bovine serum and 1% of penicillin and streptomycin. After 48 h, 20 uL MTT was added to absorb culture medium. Four hours' later, the supernatant was discarded after centrifugation. 150 uL DMSO was added to terminate the reaction. The absorbance at 490 nm was detected by ELIASA and the cell activity was detected comparing with the absorbance of control hole.

Cell relative proliferation activity (%) = absorbance of experimental group/absorbance of control group $\times 100\%$.

2.2.5. Apoptosis rate

1.5 × 10⁵/L tumor cells were inoculated in culture flasks and washed two times with PBS. Then 100 uL Binding Buffer and 5 uL Annexin-V (20 ug/mL) marked by FITC were added. They were gently mixed together and incubated for 15 min at 4 °C without light, then added 5 uL PI (50 ug/mL) into them. After reacting for 5 min without light, 400 uL Binding Buffer was added into. Apoptosis rate (within 1 h) were detected by flow cytometry (model: EPICS-ELITE-ESP).

2.3. Experimental ethics statement

All experiments in the study were conducted according to the guidelines on animal healthcare and use of animal experimental center of Zhengzhou University in Henan Province and approved by local ethics committee and conformed to the guidelines on healthcare and use of experimental animals made by National Institutes of Health (Publication No. 85 – 23, revised in 1985).

3. Results

3.1. Comparing the efficacy in each group

Ten days after implantation tumor model, 100 mm³ tumor had been founded under the skin of mice. After different treatment, the changes of tumor volume in each group were shown in Table 1. From the table, we could see that the tumor volume of routine chemotherapy group and curcumin chemotherapy group were significantly smaller than that of the control group (P < 0.05). 28d later, all mice were sacrificed and the tumor tissues were stripped and weighed. The tumor weight of control group was (0.75 ± 0.36) g, the tumor weight of routine chemotherapy group was (0.27 ± 0.11) g, and the inhibition rate was 63.8%; the tumor weight of curcumin chemotherapy group was (0.14 ± 0.07) g, and the inhibition rate was 81.3%.

The content of vascular endothelial growth factor (VEGF) was closely related to the prognosis of tumor. The serum VEGF content of each group had no significant differences before treatment (P > 0.05). The VEGF content of each group at different times were shown in Table 2. The serum VEGF content of chemotherapy group and VCR chemotherapy group were significantly lower than control group after 2-week's treatment. 4 weeks later, the VEGF content of chemotherapy group and curcumin chemotherapy group were lower than control group, and there was significant difference between first two groups (P < 0.01).

3.2. The cell proliferation activity and apoptosis rate in each group

The cell proliferation activity and apoptosis rate were detected by MTT and flow cytometry method (Table 3). We could see that the proliferation activity of chemotherapy group and curcumin chemotherapy group was significantly lower than control group, but the apoptosis rate of them was higher than control group. The effect of inhibiting tumor cell proliferation on curcumin chemotherapy group was more obvious than routine chemotherapy group.

Table 1
Tumor volume of each group at different times (Mean ± SD, mm³).

Group	1 d	4 d	7 d	10 d	13 d	16 d	19 d	22 d	25 d	28 d
Routine chemotherapy group	104.4 ± 19.6	135.6 ± 21.2*	166.3 ± 23.6*	201.3 ± 26.8*	246.2 ± 20.4*	273.2 ± 21.5	283.5 ± 24.6*	280.4 ± 26.8*	289.6 ± 22.4*	292.3 ± 26.4*
Curcumin chemotherapy group	101.9 ± 12.5	123.5 ± 17.3*	152.3 ± 21.2*	181.6 ± 23.4*	214.3 ± 23.5*	220.2 ± 24.3*	201.3 ± 23.1*	187.3 ± 22.4*	164.2 ± 26.4*	155.2 ± 25.1*
Control group	102.7 ± 19.4	324.5 ± 31.4	553.6 ± 33.1	653.6 ± 46.5	746.4 ± 57.8	787.4 ± 51.1	813.6 ± 54.6	851.2 ± 54.6	872.4 ± 52.6	889.3 ± 56.7

*P < 0.05 compared with control group; ∇P < 0.05 compared with routine chemotherapy group; †compared with routine chemotherapy group, ‡ = 15.732.

Table 2VEGF content of each group at different times (Mean \pm SD, ng/mL).

Group	7 d	14 d	21 d	28 d
Control group	575.63 \pm 28.32	586.34 \pm 29.34	601.32 \pm 30.14	616.24 \pm 21.24
Coutine chemotherapy group	403.54 \pm 19.63*	352.34 \pm 17.21*	291.63 \pm 16.32*	234.31 \pm 31.24*
Curcumin chemotherapy group ¹	384.51 \pm 18.62*	303.67 \pm 15.21* ∇	153.67 \pm 8.21* ∇	86.45 \pm 5.23* ∇

* $P < 0.05$ compared with control group; $\nabla P < 0.05$ compared with routine chemotherapy group; ¹compared with routine chemotherapy group $\chi^2 = 26.374$.

Table 3

The cell proliferation activity and apoptosis rate in each group (%).

Group	Proliferation index	Apoptosis rate
Routine chemotherapy group	15.69 \pm 5.21*	22.37 \pm 8.96*
Curcumin chemotherapy group	11.57 \pm 4.25* ∇ ¹	37.23 \pm 12.68* ∇ ²
Control group	33.74 \pm 10.68	14.21 \pm 5.14

* $P < 0.05$, compared with control group; $\nabla P < 0.01$ compared with routine chemotherapy group; ¹compared with routine chemotherapy group; $\chi^2 = 10.542$; ²compared with routine chemotherapy group $\chi^2 = 9.427$.

4. Discussions

Preoperative adjuvant chemotherapy is an effective treatment to reduce the surgical risk of nephroblastoma and obtain good prognosis. But because of drug resistance of tumor cells, sometimes the conventional chemotherapy is hard to obtain satisfactory effect [11]. Curcumin is a kind of polyphenolic compounds extracted from zingiberaceae plants [12]. Surveys and experimental researches showed that Curcumin has a variety of clinical medical value in inflammation, cardiovascular and digestive system, and plays a positive role in resisting the occurrence and development of tumor [13,14]. Curcumin can not only inhibit the growth of many tumor cells, but also enhance the sensitivity of chemotherapy and radiotherapy of breast cancer cells, cervical cancer cells *etc.* [15,16]. Studies have confirmed that curcumin also can enhance the chemosensitivity of malignant glioblastoma and neuroblastoma, and prevent cancer cells from transferring to other parts and promoting apoptosis [17], but the researches on curcumin which are used for chemotherapy of nephroblastoma cells are insufficient. This study used curcumin combined with vincristine and actinomycin D to treat neuroblastoma. The results showed that after adding curcumin chemotherapy, curcumin on inhibiting tumor growth in mice was more significant than routine chemotherapy group ($P < 0.05$ or 0.01). In addition, we found that the serum VEGF content of tumor-bearing mice in curcumin chemotherapy group was less than routine chemotherapy group by detecting the VEGF content as tumor marker in tumor bearing mice ($P < 0.05$). And after 4-week's treatment, the serum VEGF content of curcumin chemotherapy group was close to normal mice.

The specific mechanism of curcumin on tumor has not been clearly studied yet. Now, the basic conclusion we have reached is that this material can promote the generation of fast reactive oxygen in cells, promote cells secreting lots of apoptosis inducing factor (AIF), activate the apoptosis pathway of Caspase-3, and inhibit the malignant proliferation of cancer cells effectively [18]. Chang *et al.* [19] also found that curcumin

could activate signaling pathway of ROS/Cyto-C/Caspase-3 and promote osteosarcoma cell MG63 tending to apoptosis. Ai *et al.* [20] confirmed that the mechanism of activating Caspase signaling pathway by curcumin is to stimulate mitochondria secreting AIF in cells and promote tumor cells such as melanoma tending to apoptosis. In addition, Yang *et al.* [21] found that curcumin could reduce the expression content of tumor protein STAT3 and IL-6, inhibit the malignant proliferation and metastasis of non small cell lung cancer. Li *et al.* [22] found that curcumin could down regulate oncogenic gene MDM2 by PI3K/mTOR/ETS2 signaling pathway, up regulate the expression of anti oncogene p21, and play a role in anti-tumor and enhancing the sensitivity to chemotherapy and radiotherapy of tumor cells. Fan *et al.* [23] found that curcumin was the inhibitor of fatty acid synthase (FAS), and played a role in anti-tumor and enhancing the chemosensitivity by inhibiting the expression of tumor protein in liver cancer. Therefore, it could be seen that the curcumin in different tumor tissues were related to the apoptotic pathway, but its anti-tumor effect modes were different. Therefore, the specific mechanism which curcumin increases chemosensitivity of nephroblastoma cells still need to be studied and confirmed by relevant molecular experiments.

In summary, for nephroblastoma, curcumin has a unique antitumor effect, and plays a synergistic role with routine chemotherapy drugs. The mechanism for enhancing chemosensitivity of cells is to activate the related apoptosis pathway. In the research, tumor bearing mice were given curcumin by intraperitoneal injection which enhanced the chemotherapy effect of conventional chemotherapy drugs such as vincristine, but in actual clinical trial, further studies were needed to make sure suitable dosage of curcumin to anti tumor and related side effects; Therefore, in further experiments, we need to design different curcumin doses or courses of treatment, to seek the best preoperative auxiliary chemotherapy for nephroblastoma, reduce the surgical risk and improve the prognosis effect.

Conflict of interest statement

We declare that we have no conflict of interest.

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