The Inhibitory Effect of Carboplatin Injection on Human Neuroblastoma SK-N-SH

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Abstract

The purpose of our study was to investigate the inhibitory effect of carboplatin injection on human neuroblastoma human neuroblastoma cell (SK-N-SH) cells and to clarify its action mechanism. In this study, human neuroblastoma SK-N-SH cells were divided into two groups. The treatment group was intervened by carboplatin injection ($25 \mu M$), while the control group was intervened by drug solvent. After treating separately for 24 and 72 h, the cells were collected, and western blot (WB) and real-time PCR were used to detect the expression of the proliferation marker protein (Ki67); cells grown on cover slips were prepared and immunocytochemistry (ICC) and hematoxylin–eosin (HE) staining were adopted to observe the protein expression of Ki67 and the morphological changes of the cells; clone formation assay was used to detect the clonality of each cell group. The cytotoxicity of carboplatin on SK-N-SH cell was checked by AlamarBlue viability test. Both WB and PCR results showed that after cells were injected with carboplatin for 24 and 72 h, the expression levels of both Ki67 gene and protein were decreased, and they had a significant difference from those of the control group. Carboplatin injection inhibited the expression of Ki67, and the inhibitory effect was particularly significant as the action time prolonged. ICC results showed that the protein expression of Ki67 in the treatment group was lower than that in the control group, and there was a significant difference in expression between them. As shown by HE results, the number of cell necrosis and apoptosis in the treatment group was significantly higher than that in the control group, while the results of clone formation assay showed that in the treatment group, after being injected with carboplatin, the proliferation ability of cells was inhibited, so the number of cells was significantly reduced compared with that of the control group. Carboplatin at the tested concentration displayed no cytotoxicity on SK-N-SH cell. The conclusions are that carboplatin injection can inhibit human neuroblastoma SK-N-SH cells, and the longer it acts on SK-N-SH cells, the more obvious the inhibitory effect would be.

Keywords

carboplatin injection, human neuroblastoma SK-N-SH, Ki67

Introduction

Neuroblastoma, as one of the most common malignant tumors in children, accounts for 8%–10% tumors in children. The incidence of infants aged below 2 suffering from neuroblastoma SK-N-SH is 50%, so it has a high mortality rate[1,2]. Neuroblastoma is a type of cancer developed from immature nerve cells of the body, which mostly originate from the sympathetic nerves of the chest and abdomen. The symptoms of this disease are abdominal distension, abdominal pain, vomiting, anorexia, weight loss, fatigue, and so on. Although there are currently many therapeutic schemes for neuroblastoma such as surgical resection, radiotherapy, and chemotherapy, the survival rate of children is still low, so it poses a serious threat to the health of infants[3–8]. It has been found that clinical drugs used for treating other tumors may play a role in the treatment of neuroblastoma.

Carboplatin injection used for clinical treatment has a good curative effect[9] on some solid tumors including ovarian cancer, nonsmall cell lung cancer, and head–neck carcinoma. According to literature reports, carboplatin injection

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Table 1. Primer Sequence.

Primer name	Forward primer (5′–3′)	Reserve primer (5′–3′)
Ki67	GCAGGACTTCACTTGCTTCC	TCATTTGCGTTTGTTTCAC

has a certain effect on other tumor cells[10–14]. In this study, a certain concentration of carboplatin injection was used to intervene with human neuroblastoma cells, and by detecting the expression of proliferation marker protein Ki67 as well as by testing the proliferation ability of cells through clone formation assay, it was found that carboplatin injection had a certain inhibitory effect on neuroblastoma[15–19]. It laid a foundation for the follow-up mechanism study and a theoretical basis for the future cure of neuroblastoma.

Materials and Methods

Ethical Approval

The study was approved by the Institutional Ethics Committee of West China Hospital.

Cell Culture

SK-N-SH cells were purchased from the Cell Resource Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified eagle medium (DMEM, 12100-46; Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum was cultured in an incubator at 37°C with 5% CO_2[20–22]. The specification of carboplatin injection is 10 mL:100 mg (Qilu Pharmaceutical Co., Ltd, Jinan City, Shandong Province; batch no. H20020180). SK-N-SH cells were divided into two groups. The treatment group was injected with 25 μM carboplatin to intervene with cells while the control group was treated with drug solvents. After being treated for 24 and 72 h, respectively, cells were collected for subsequent experimental detection.

Real-Time Polymerase Chain Reaction (PCR)

Real-time polymerase chain reaction (PCR) was used to detect the gene expression level of Ki67 in different groups, and TRIpure Extraction Kit (RP1001; BioTek, Beijing, China) was adopted to extract the total amount of ribonucleic acid (RNA) in each cell group according to instructions. Nanodrop-2000 (Thermo Fisher Scientific, MA, USA) was used to detect the RNA concentration in each group. Reverse transcription was carried out using real-time PCR (Exicycler 96; BIONEER, Republic of Korea) and Super M-MLV reverse (PR6502; BioTek). Then, real-time PCR was done by using $2\times$ power Taq PCR mastermix (PR1702; BioTek) and SYBR Green I (SY1020; Solarbio, Beijing, China). Based on the internal parameter of β-*actin*, the relative messenger RNA (mRNA) expression level of Ki67 was calculated through the formula $2^{-\Delta\Delta Ct}$[23]. The primer sequence is shown in Table 1, which was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

Western Blot

The cells were collected and lysed on ice for 5 min with radio immunoprecipitation assay (RIPA) lysis buffer (P0100; Solarbio) containing 1 mM phenylmethane, then centrifuged at 12,000 rpm, 4°C for 10 min, and from the separated supernatant protein was extracted. BCA Protein Assay Kit (PC0020; Solarbio) was used for protein quantification. According to the experimental process of western blot (WB), the protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membrane (IPVH00010; Millipore, MA, USA). It was sealed with 5% skimmed milk. After adding Ki67 antibody (1:500 dilution; WL01384a; Wanleibio, Shenyang), it was incubated overnight at 4°C. After cleansing with tris buffered saline tween (TBST), it was incubated with horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG (1:3000 dilution; SE134; Solarbio). So it was the same process with the internal parameter β-*actin*. Finally, the electrochemiluminescence (ECL) luminescent liquid (PE0010; Solarbio) was added and exposed in a dark room. The film was scanned and the optical density of the target strip was analyzed by gel image processing system (Gel-Pro-Analyzer, State of California, USA).

Immunocytochemistry

As the cells were collected, cells grown on cover slips were prepared and fixed with 4% paraformaldehyde solution. Formaldehyde was removed and 0.1% Triton X-100 was added for incubation for 20 min. After removal, 0.01 M PBS was added and soaked for 5 min three times. Then, phosphate buffer saline (PBS) was removed and serum was added and sealed for 15 min. After the serum was removed, Ki67 primary antibody diluted with PBS at 1:200 (1:300 dilution; WL0280a; Wanleibio) was added for overnight incubation at 4°C. Then, the primary antibody was removed, 0.01 M PBS was added to them, and soaked for 5 min three times.

Afterwards, PBS was discarded; PBS was dripped to dilute HRP-labeled goat anti-rabbit secondary antibody 100 times (1:100 dilution; WLA023a; Wanleibio) until it fully covered the cells. Then the cells were incubated at 37°C for 60 min. After removing the secondary antibody, the cells were soaked in 0.01 M PBS for 5 min three times. Next, PBS was removed and diaminobezidin (DAB) was added for coloration; then hematoxylin was used for restaining. The staining effect was observed and photographed under a microscope.

Hematoxylin–Eosin (HE) Staining

Cells grown on cover slips were prepared, fixed with 4% paraformaldehyde solution for 15 min, and then soaked in distilled water for 5 min three times. Hematoxylin was added and the cells soaked in it for 5 min before being soaked in distilled water for 5 min. Next, they were allowed to stay in 1% hydrochloric acid alcohol for 3 s. After cleansing with distilled water, eosin dye solution was added to them and soaked for 3 min. After eosin staining, 75%, 85%, and 95% concentration levels of ethanol were added in turn, each for 2 min. The staining effect was observed and photographed under the microscope.

Colony Formation Assay

SK-N-SH cells were cultured to a density of about 90% by trypsin enzyme–digesting technique and centrifuged at 1000 rpm for 5 min to collect the cell precipitation. The supernatant was added into the complete medium, and cells were then resuspended. The cells were inoculated according to experimental grouping. The cells in the treatment group were treated with 25 μM carboplatin injection and collected after 24 and 72 h, respectively. The cells of each group were inoculated in the culture dish, each dish inoculated with 400 cells, and the culture dish was cultured at 37°C and 5% CO_2. It took about 2 weeks to form clones that could be seen by naked eyes, and then they could be fixed. They were washed with PBS twice and fixed with 4% paraformaldehyde at room temperature for 20 min. Then they were washed with PBS twice, and Richter's compound dye was used for dying for 5 min. Then, they were washed with water three times before scanning. Under the microscope, the cell mass made of 50 or more cells is the number of clones. Clone formation rate = (number of clones/number of inoculated cells) × 100%[24].

Cytotoxicity Tests for Carboplatin on SK-N-SH cells

The cytotoxicity of carboplatin was checked by AlamarBlue viability test which is especially important for the protection of functional effects. The number of living cells was determined indirectly by determining the mitochondrial metabolic activity of the cells under this test. The cells were seeded into duplicate wells of a 48-well plate techno plastic products (TPP) at a density of 1500 cells/mL and precultured under standard conditions (5% CO_2, 37°C) for 3 days. Subsequently, cells were treated with 25 μM carboplatin for 24 and 72 h, respectively. Then the cells in each well were seeded with 360 μL medium with 10% AlamarBlue and grown at 37°C under 5% CO_2 for 4 h. For control, AlamarBlue was added to untreated cells as well as to medium without cells. The absorbance at 570 and 600 nm were measured by microplate photometer. The viability of the cells was then calculated by using the formula in a previous study[25]. Viability of the cells=(ODtreated—ODblank/ODcontrol—ODblank)×100%.
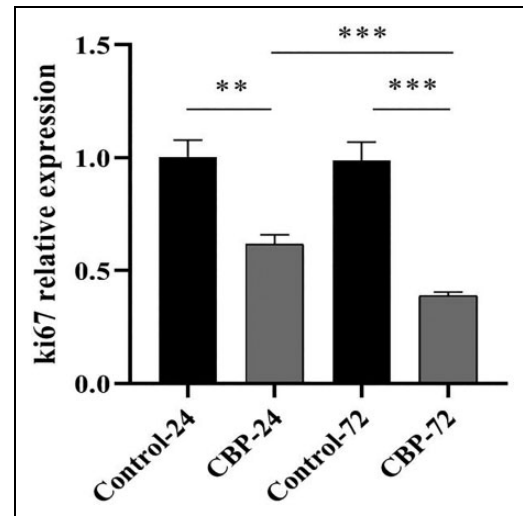

Fig. 1. The gene expression of Ki67 in different treated cells. There was a significant difference between control-24 and CBP-24 (**$P <$ 0.01); CBP-72 has a highly significant difference from control-72 and CBP-24 (***$P < 0.001$). CBP: carboplatin.

Statistical Analysis

All data are expressed as mean $\pm$ standard deviation. SPSS 20.0 and GraphPad Prism 8.0 were used for statistical analysis and mapping. T-test was conducted by means of one-way analysis of variance (ANOVA) and multiway ANOVA. When the value of P is less than 0.05, it is considered statistically significant (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Results

The Gene Expression Level of Ki67 in the Treatment Group Was Lower Than That in the Control Group

A real-time PRC experiment was carried out in order to study the gene expression of Ki67 in SK-N-SH cells treated with carboplatin injection. The results showed that the gene expression level of Ki67 decreased significantly after being treated for 24 h, and the inhibition effect of Ki67 gene grew more obvious after being treated for 72 h with a highly significant difference; as the action time of carboplatin injection prolonged, the gene expression level of Ki67 in 72 h was much lower than that in 24 h with a highly significant difference (Fig. 1).

The Protein Expression of Ki67 in the Treatment Group Was Lower Than That in the Control Group

In order to better clarify the effect of carboplatin injection on the protein expression of Ki67 in cells, WB was used to detect the protein expression level. The results showed that the protein expression level decreased significantly after the treatment of carboplatin injection, and the longer it acted on
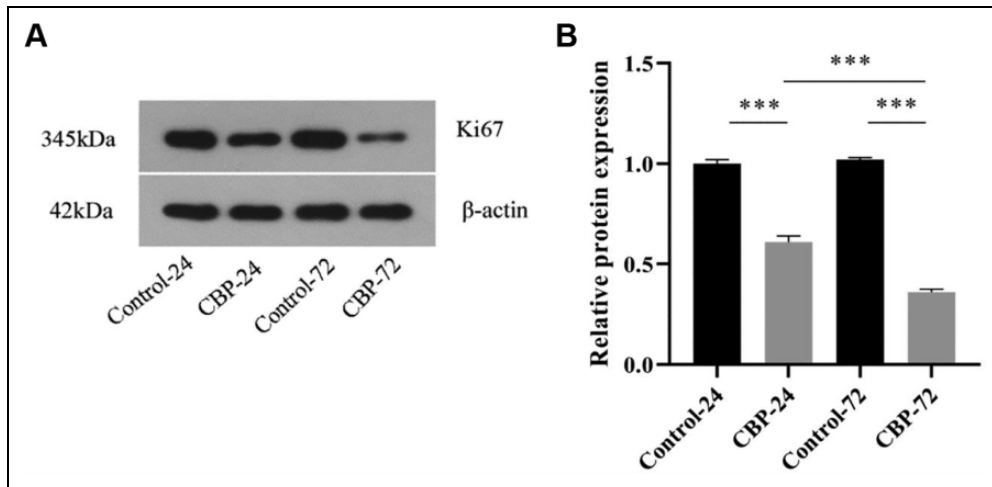
Fig. 2. The effect of different treatments on the expression of Ki67 protein. The expression level of the control group and the treatment group at the same time point and the expression level of CBP-24 and CBP-27 both had a highly significant difference (***P < 0.001). CBP: carboplatin.
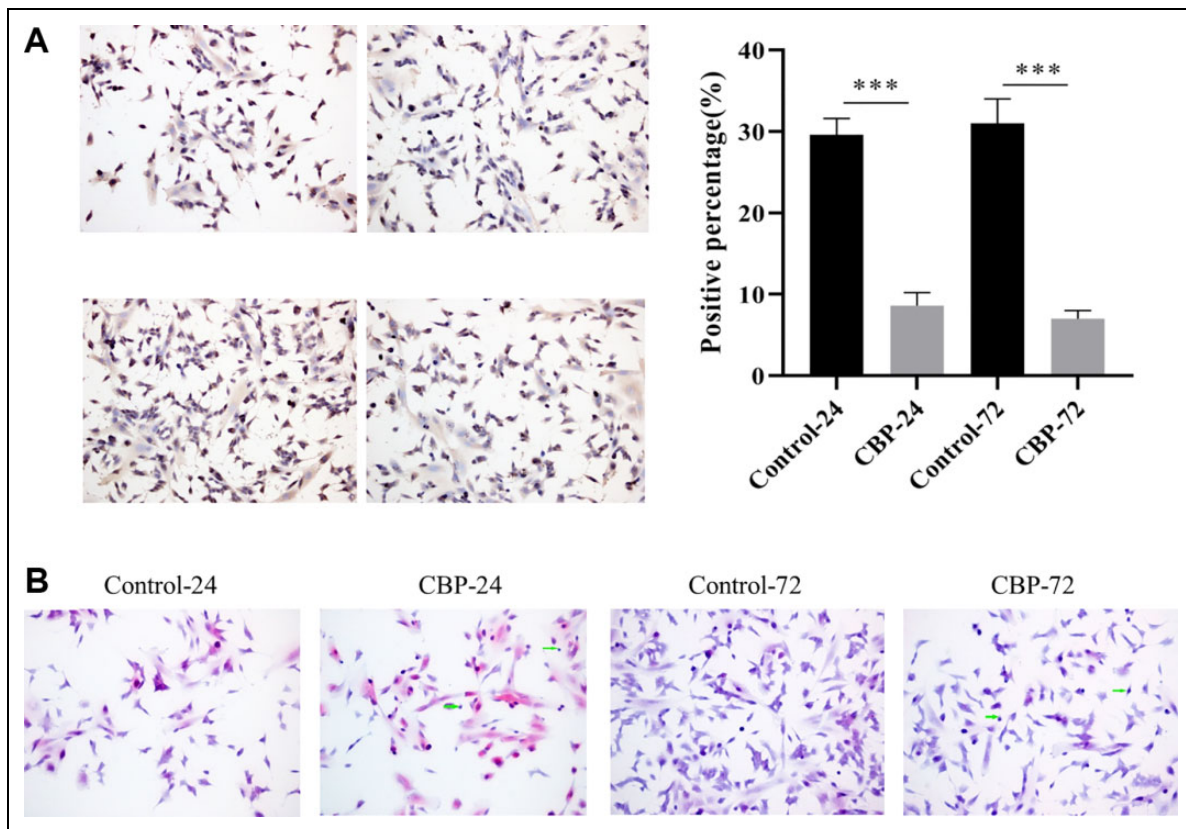

Fig. 3. Pathological results after the treatment of SK-N-SH cells with carboplatin injection. (A) The ICC results showed that there was a highly significant difference in the expression of Ki67 between the treatment group and the control group (***P < 0.001); (B) after being injected with carboplatin, the cell apoptosis and necrosis were significantly higher than those of the control group. CBP: carboplatin.
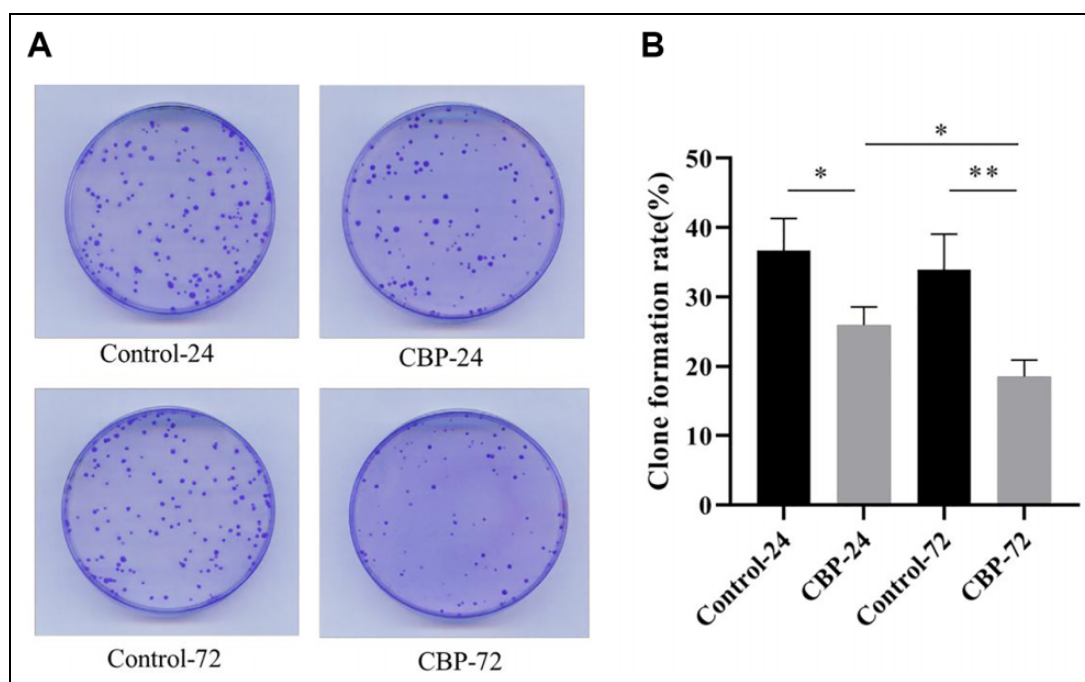
Fig. 4. The effect of carboplatin injection on the proliferation of SK-N-SH cells. (A) Pictures of cloning formation assay; (B) There was a significant difference between control-24 and CBP-24 and between CBP-24 and CBP-72 (*$P < 0.05$); there was a significant difference between CBP-72 and control-72 (**$P < 0.01$). CBP: carboplatin.

the protein, the more obvious the effect was, and the more significant the difference would be (Fig. 2A, B).

ICC Results Showed That the Protein Expression of Ki67 in the Treatment Group Was Lower Than That in the Control Group, and HE Results Showed That the Drug Could Promote Cell Necrosis and Apoptosis

After cells were injected with carboplatin, cells grown on cover slips were prepared. ICC and HE staining were used to observe the protein expression of Ki67 and the morphological changes of the cells. By analyzing the positive percentage of cells in ICC results, it was found that the protein expression of Ki67 in the treatment group was significantly lower than that in the control group and the difference was highly significant (as shown in Fig. 3A). The results of HE staining showed that the cells in control-24 were the same in form and size, while some cells in the carboplatin (CBP)-24 were necrotic or even apoptotic; the cells in control-72 had a high density in a same form, and there were more necrotic cells in the treatment group than in the control group. The necrotic cells are pointed out with green arrows in Figure 3B.

Carboplatin Injection Reduces the Clone Formation Ability of Cells

After treating SK-N-SH cells with different methods, clone formation assay was used to detect the proliferation
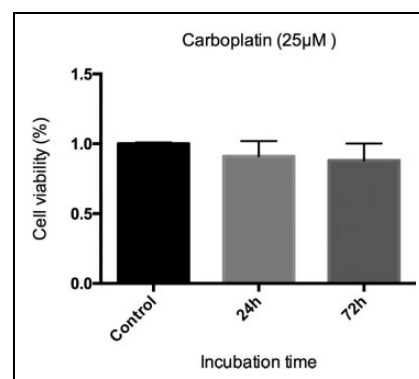

Fig. 5. AlamarBlue assay of Carboplatin on SK-N-SH cell.

ability of the cells. The results showed that after being treated for 24 and 72 h, carboplatin injection inhibited and weakened the proliferation of cells. Statistical analysis of the results showed a significant difference, and the effect of 72-h treatment was more obvious than that of 24-h treatment (Fig. 4A, B).

Cytotoxicity Tests for Carboplatin on SK-N-SH Cells

It was demonstrated by the AlamarBlue assay that carboplatin at a concentration of 25 μM displayed no cytotoxicity on SK-N-SH cells after 24 and 72 h incubation (Fig. 5).

Discussion

In this study, a certain concentration of carboplatin injection was used to intervene with human neuroblastoma SK-N-SH cells. The results in all these aspects showed that carboplatin injection had a significant inhibitory effect on neuroblastoma SK-N-SH. Literature review found that carboplatin injection also plays a similar role in inhibiting other tumors. It can inhibit the proliferation of nonsmall cell lung cancer and facilitate its apoptosis[14]. In the treatment of retinoblastoma (RB), carboplatin injection also plays a particularly inhibitory role. Similar to RB, neuroblastoma SK-N-SH is also a common disease in children, most of whom are under 3 years, and it has a family genetic tendency. RB is a common malignant tumor in infants and young children, but rarely seen in adults. Carboplatin injection plays an important role in curing RB, saving children's eyes and reducing infant mortality[26].

At present, Ki67 gene is a relatively positive cell proliferation marker. Ki67 was found by Gerdes[27] et al. in 1983. Since then it has been proved that Ki67 gene is closely related to cell proliferation and tumor treatment, and it has gradually become a common indicator to judge the degree of malignancy and the prognosis of tumors. In cell cycle, it exists in all other phases as an essential part of cell cycle regulation except for G0 phase. It has been reported that Ki67 is strongly correlated to urinary system tumors. Kamai[28] and Korkolopoulou et al.[29] after observing the relationship between Ki67 and the malignant degree of bladder cancer came to believe that Ki67 gene played a significant role in tumor screening. By studying the relationship between Ki67 and renal cancer, Pich and Deriese found that the grading of tumors was positively related to the expression of Ki67-positive cells, and that the proliferation index of Ki67 was closely related to the prognosis of renal cancer. In addition, there is plenty of literature showing that Ki67 is related to breast cancer, rectal cancer, pancreatic neuroendocrine tumors, and so on. Deriese and Crabtree[30] et al. studied the relationship between Ki67 and renal cancer tissues and found that the degree of tumor grade was positively correlated with the expression of Ki67-positive cells, and the proliferation index of Ki67 was closely related to the prognosis of renal cancer. In addition, there is a large amount of literature showing that Ki67 is related to breast cancer[31–33], rectal cancer[34], and pancreatic neuroendocrine tumor[35].

In this study, it is found that the mRNA and protein expression levels of Ki67 were decreased after the cells were treated with carboplatin injection, both of them being significantly different. Besides, the longer carboplatin injection acted on cells, the more obvious the effect would become. Moreover, clone formation assay also showed that the proliferation ability of the cells injected with carboplatin was significantly weakened, which is of statistical significance compared with that of the control group. By comparing 72 and 24 h, we can clearly see the time dependence of neuroblastoma SK-N-SH cells on the drug effect. It is believed that the effect of carboplatin will be more obvious as the time increases.

It is speculated that after cells were injected with carboplatin, it is Ki67 that played the role of medium, and then regulated the expression of intracellular related pathway proteins, thus leading to functional changes in cells and inhibiting neuroblastoma SK-N-SH. In the follow-up study, effort would be made to further explore the involved signaling pathways and the mechanism by which carboplatin injection played the role of inhibiting tumors. It is believed that with the deepening of research, the action mechanism will be better clarified and play a greater role in the follow-up clinical application.

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