

IMPROVED FREEZING INJURY OF IN VITRO BRAIN TUMOR CELLS BY USE OF NANOPARTICLE ADJUVANTS

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ABSTRACT

Along with the development of modern imaging techniques, cryosurgery is emerging as an important minimally invasive surgical method for treatment of brain tumors. Although imaging allows excellent control over the freezing extent, enhancement of cryosurgical injury of tumor cells at freezing temperature is also preferred to improve cryosurgical outcome. The goal of this study is to find effective cryosurgical adjuvants to enhance freezing injury of brain tumor cells. Considering the important role of ice crystal formation in freezing induced cell injury, nanoparticles were introduced in this study to produce more ice crystals and thus to enhance freezing injury of tumor cells. Cellular suspensions of brain tumor with and without nanoparticles were respectively frozen using a cryosurgical apparatus with two freeze-thaw cycles. The viability of brain tumor cells was then assessed by typan blue dye exclusion. The results indicate that nanoparticles significantly increase cellular destruction. It suggests that nanoparticles may be effective adjuvants to cryosurgical treatment of brain tumors.

1. INTRODUCTION

Brain tumor patients, including those with certain benign brain tumors, have lower survival rates than other cancer patients (Stafinski *et al.*, 2006). There are over 120 different types of brain tumors, making effective treatment very complicated (Meyerand *et al.*, 1999). At present, brain tumors are routinely treated by surgical excision (Raco *et al.*, 2004), radiotherapy (Stafinski *et al.*, 2006) and chemotherapy (Buckner, 1991; Duffner *et al.*, 1993), used either individually or in combination. Since brain is the control center for thought, emotion and movement, the possible side-effects of conventional surgery, radiotherapy and chemotherapy sometimes may be devastating when brain tumors are located at these key function areas.

Cryosurgery is a minimally invasive surgical procedure that makes use of local freezing for the controlled destruction of diseased tissues (Baust, *et al.*, 1997). The use of cryosurgery in these cases often appears as an attractive choice. In fact, at the beginning of modern era of cryosurgery (1960's), the use of cryosurgery for treatment of brain tumors was ever of special interest to neurological surgeons (Cooper and Lee, 1961; Cooper and Stellar, 1963; Hamlin, 1969; Walder, 1975). Because neuroimaging techniques were not well developed at 1960's, brain cryosurgery had not widely accepted, and received few attentions about two decades from that time.

At 1990's, advances in neuroimaging and cryosurgical techniques have prompted researchers to re-evaluate the potential of cryosurgical techniques for the removal and the destruction of various brain tumors (Maroon

et al., 1992; Maroon and Bailes, 1995). In 1992, Maroon and his colleagues (Maroon *et al.*, 1992) reported the cryosurgical treatment of 71 patients with diverse types of brain tumors. The cryosurgical procedures were successful, at least judged in a short-term follow-up. In addition, results from other report also suggested that cryo-assisted treatment of brain tumors is an effective technique (Endo *et al.*, 1993).

The aim of cryosurgery is to exactly kill all tumor cells within a closely defined region. This is particularly true in brain cryosurgery, where the destruction of healthy tissue may result in loss of some key functional or mental abilities. Therefore, enhancement of cryosurgical injury of tumor cells at freezing temperature is preferred to improve the outcome of brain cryosurgery, although neuroimaging has made it possible to control the freezing to a certain extent. To enhance the freezing injury of tumor cell, the use of adjuvants such as cancer chemo-therapeutic agents and antifreeze protein has been proved to be feasible (Clarke *et al.*, 2001; Pham *et al.*, 1999). Recently, we also proposed a new method using nanoparticles with high thermal conductivities to improve the efficacy of cryosurgery (Yu *et al.*, 2005; Deng and Liu, 2005), which has been proved to be effective from the mechanism of heat transfer. In fact, another possible mechanism of enhancement of freezing injury may be associated with more ice crystals (including both extracellular and intracellular) produced by nanoparticle.

Based on the above consideration, nanoparticles were introduced in this study to enhance freezing injury of tumor cells by producing more ice crystals. In order to prove the feasibility of this approach, experiments were performed on freezing of cellular suspensions of brain tumor with and without nanoparticles, respectively. Cell death rates with the corresponding freezing experiments were then compared.

2. MATERIALS AND METHODS

2.1. Sample Preparation

Human glioma cell line SF763, obtained from the Cancer Institute & Hospital of Chinese Academy of Medical Sciences, was cultured in standard medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 37°C in a humidified 95% air and 5% CO₂ atmosphere. Before experiments, cells were detached from the culture flask and washed with 0.25% Trypsin solution and phosphate buffered saline (PBS). The cells suspended in PBS were then divided into 3 groups. Group 1 was the control group, in which no nanoparticles were loaded. Group 2 and group 3 were treated with carbon nanotube (CNT) and silver nanoparticles (SNP), respectively. After this, cells in group 2 were suspended in a solution of 2mg/ml SNP, and cells in group 3 were suspended in a solution of 2mg/ml CNT.

2.2. Freezing Experiments

The freezing experiments on cell suspensions were carried out by using a liquid-nitrogen-based cryosurgical system, developed by the Technical Institute of Physics and Chemistry of Chinese Academy of Sciences (Liu *et al.*, 2004). Samples were prepared in 10ml freezing tubes. A 5mm diameter cryoprobe was particularly selected to perform the simulated cryosurgical process, and two freeze-thaw cycles were conducted. During experiments, the cryoprobe was directly inserted into the freezing tubes. To avoid overflow of the solution of cellular suspension from the tube during freezing, 5ml suspension was used for each group to perform freezing experiment.

The general protocol of the freezing experiment was as follows. First, a sample was cooled for 8 minutes by

the cryoprobe. Second, freezing was temporarily stopped. After 7 minutes thawing naturally in the air, the sample was cooled once again for 5 minutes. Freezing was then stopped, and the frozen sample was thawed in an isothermal water bath. Finally, cell viability of the sample after two freeze-thaw cycles was tested. During experiments, temperature of the sample was measured with copper-constantan thermocouples connected to an Agilent 34970A Data Logger. The thermocouples were calibrated before experiments and an accuracy of $\pm 0.1^\circ\text{C}$ was obtained.

2.3. Evaluation of Cell Death Rate

Using a biological microscope (DM-IRB, Leica), cell viability was assessed by typan blue dye exclusion. The viability was calculated from the numbers of all cells appearing with intact membrane integrity. Cell death rate was defined by the ratio between the viability difference (before and after experiments) and the viability before the experiments.

3. RESULTS AND DISCUSSION

Figure 1 shows the temperature responses of the cellular suspensions during freezing and thawing, in which group 1 is the case without introducing nanoparticles, and group 2 denotes the case of adding CNT into cell suspension while group 3 is the case of treating the cell suspension with SNP. The temperature measurement positions for all cases were at a distance of 1mm from the tip of cryoprobe. The data of temperature responses given in Fig. 1 indicate that once frozen by the cryoprobe, the temperatures of cellular suspensions could decrease quickly until they reach about -180°C and are stabilized there. Generally, this takes time less than 300s, as shown in Fig. 1. By comparing the results depicted in Fig. 1 for the three different cases, it can be easily found that the temperatures of cell suspensions for the cases of treating with nanoparticles (group 2 and group 3) drop more quickly than that for the case without treating with nanoparticles (group 1) during freezing, and that the temperature for the case treating with SNP drops the most quickly among the three groups. It indicates that from the point of heat transfer mechanism, introducing nanoparticles with high thermal conductivities to improve the freezing effect is feasible, as has been demonstrated in our previous study (Deng and Liu, 2005).

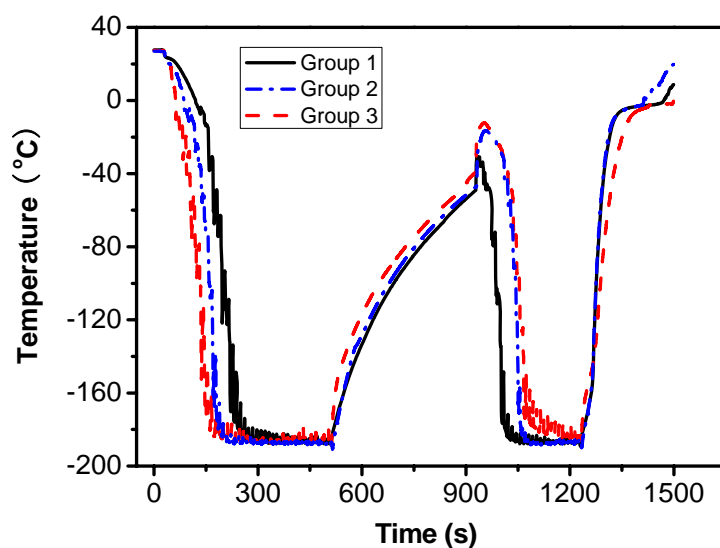


Figure 1. Temperature responses of the cellular suspensions during freezing and thawing.

Figure 2 depicts the percentage of dead cells respectively for the three different cases, following freezing by the same cryoprobe with same freezing power. An obvious difference in cell death rates can be found between the cases with and without introducing nanoparticles into the cellular suspensions. The results show that in the absence of nanoparticles, a significant percentage (about 18%) of tumor cells survive freezing. Only about 6% of cells survive when the sample is treated with CNT, and all cells are completely destroyed when treated with SNP. It indicates that by introducing nanoparticles, freezing injury to brain tumor cells has significantly increased, and that using nanoparticles as an adjuvant to cryosurgery could result in complete destruction to tumor cell. However, if no measure has been adopted to improve the cryosurgical injury of cells, tumor recurrence may be resulted from the surviving tumor cells, although all tumor tissue is totally frozen during cryosurgical treatment.

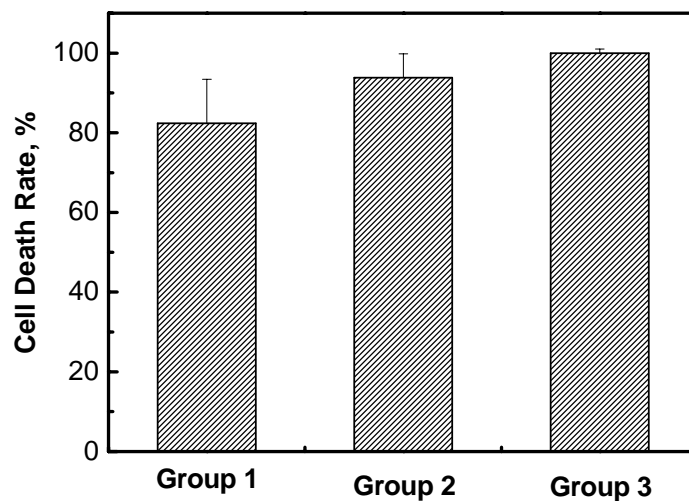


Figure 2. Percentage of cells destroyed by freezing under different conditons.

It is well known that the two main mechanisms of cell destruction during cryosurgical procedure are associated with extracellular and intracellular ice crystallization, respectively (Rubinsky, 2000). When nanoparticles are introduced into cell suspension, they will permeate everywhere within the suspension (both extracellular and intracellular). Consequently, more ice crystals will be formed in both extracellular and intracellular mediums due to the nucleation effect of nanoparticles during freezing. This may be the mechanism of the enhancement of cryosurgical injury to tumor cells by using nanoparticle adjuvants.

It should be pointed out that much work still remains to be done including a greater depth of inquiry into the molecular mechanisms of enhancement of freezing destruction, before the nanoparticles can be effectively used as adjuvant chemicals to brain cryosurgery, although this study suggests that introducing nanoparticles into undesirable tissues prior to freezing may increase the efficacy and the control over tissue destruction by freezing.

4. CONCLUSIONS

This preliminary study has demonstrated that nanoparticles have the ability to significantly increase and even generate complete freezing destruction to brain tumor cells during cryosurgical procedure. Even though, additional molecular-based mechanism of enhancement of freezing destruction exists and needs further

investigation, the results presented in this study suggest that introducing nanoparticles into undesirable tissues can possibly be used as an adjuvant therapy in connection with brain cryosurgery and may improve the efficacy of cryosurgical treatment in the near future.

ACKNOWLEDGMENTS

The authors thank the National Science Foundation of China for financial support of this research (No.50576104 & 50325622).

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