RESEARCH COMMUNICATION

# Induction of Apoptosis by a Combination of Paclitaxel and Carboplatin in the Presence of Hyperthermia

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## Abstract

**Purpose: To study enhancing effects of paclitaxel in the thermochemotherapy of osteosarcoma cell lines and related mechanisms. Materials and Methods: Paclitaxel and carboplatin were used alone or jointly on OS732 cell lines in the presence of hyperthermia. Inhibition of proliferation was measured by MTT assay and cellular changes were assessed with inverted phase contrast and fluorescence microscopy. Apoptosis was analyzed with flow cytometry (FCM) and Fas expression by immunocytochemistry. Results: At 43°C, one hour after the application of 10ug/ml paclitaxel and 5μg/ml carboplatin on OS732 cells jointly, the survival rate was 15.8% which was significantly lower than with 10μg/ml paclitaxel (45.8%) and 5μg/ml carboplatin (47.7%) respectively (P<0.01). Moreover, changes of morphology and apoptotic rates indicated that the apoptosis-inducing effect of combined application was also much enhanced, as evident also regarding Fas expression. Conclusion: Paclitaxel is conducive to thermochemotherapy of osteosarcoma cell lines, possibly accomplished by up-regulation of Fas expression with induction of apoptosis.**

**Keywords:** Thermochemotherapy - paclitaxel - carboplatin - osteosarcoma

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## Introduction

Osteosarcoma is the most common malignant bone tumor which seriously influence the daily life of young people for the treatment of amputation and resulting in low survival rate. In recent years, the use of local excision of tumor tissue with limb salvage surgery, combined with neoadjuvant chemotherapy has improved patient's survival rate tremendously (Bacci et al., 2008). However, local excision is not always complete, which triggers the recurrence of the primary site, leading to the failure of treatment (Franke et al., 2010; Andreou et al., 2011) and sometimes the recurrence of the local tumor happen even after the neoadjuvant chemotherapy .Local perfusion of thermochemotherapy has long been proposed as an anti-cancer modality specifically designed for the prevention of the local recurrence after the chemotherapy (Debes et al., 2005; Goto et al., 2007; Trieb et al., 2007). At high temperatures, a large number of chemotherapy drugs approach the tumor along the main arteries around primary tumor so as to kill the tumor tissue unremoved by local operation, which contribute to the limb salvage and reduce the local recurrence of primary site (Fan et al., 2003; Shido et al., 2010).

Our laboratory has focused on identifying strategies for improving the thermochemotherapy of carboplatin on osteosarcoma. We hypothesize that the combination of paclitaxel and carboplatin in the presence of hyperthermia will be able to improve the killing effect of ostesarcoma cell lines. In addition, we suppose some molecular mechanism involves in the Fas- associated cell signal pathway. The purpose of this study is to evaluate the cytotoxic effects of combination of paclitaxel and carboplatin on osteosarcoma cell lines in the presence of hyperthermia and the related mechanism of Fas-associated death receptor pathway.

## Materials and Methods

The osteosarcoma OS732 cell line was bought from Beijing Jishuitan hospital, and RPMI-1640 powder was from the Gibco company. Trypsin, MTT and Rnase A were from Huamei biological company. Paclitaxel (Beijing concord pharmaceutical factory), carboplatin (Qilu pharmaceutical company), Enzyme meter (Bio-Rad company), FACScan flow cytometer (American BD Company), LH50A inverted phase contrast microscope (OLYMPUS), fluorescence microscope (NIKON Company).

*Cell culture and research methods*

The osteosarcoma OS732 cell line was placed in the 1640 solution with 10% fetal bovine serum and cultured in incubator at 37°C with a humidified 5%

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$CO_2$ atmosphere. The cells that entered the logarithmic growth period were selected for experiment and water bathing (numerical constant temperature water bath, temperature wave≤±0.1°C) was used to heat, set at different temperature (37°C, 40°C and 43°C) for 1h. We selected the concentration of 1 μg/ml, 10 μg/ml, 50 μg/ml, 100μg/ml for the paclitaxel group , the concentration of 1μg/ml, 5 μg/ml, 10 μg/ml, 100μg/ml for the carboplatin group, and 10 μg/ml paclitaxel with 5 ug/ml carboplatin for the combined group, meanwhile setting PBS blank control group.

*Measurement of the survival rates of tumor cells*

$5\times10^5$/ml of cells after these treatments were seeded in the 96-well plate with 200ul per well, each group for parallel 4 wells. After culture for 24 h, newly made-up 5mg/ml of MTT was added to each well, and continued to incubate at 37°C for 4 h, and then the supernatant was discarded and dissolved with 150μlDMSO. The absorptance was measured at 540nm wavelength after mixed. Survival rate of tumor cells (%) = experimental group A value/control group A value×100%.

*Observation of the morphology of apoptotic cells*

The morphology, number and adherence of tumor cells were directly observed with inverted phase contrast microscope. A cover slide was placed in the 6-well plate with OS732 cells seeded, fixed for 10min and stained with 0.5ml Hoechst33258 staining solution for 5min, and then camera-imaged with fluorescence microscope on the object slide covered by cover slide and dropped with anti-fading solution.

*Measurement of the proportion of apoptotic cells*

The digested cells were collected, washed by PBS, centrifugated and then added with 70% cold ethanol to fix over night. The cells were then centrifugated to remove ethanol, and wash twice with PBS. stained in darkness with 100ulPI staining solution at 4°C for 1h. The fluorescence intensity was measured with FACScan flow cytometer. The wavelength of activated light was 488nm, and the apoptotic rates were measured with Cell Quest analysis software.

*Immunocytochemistry to detect Fas expression of OS732 cells*

$2\times10^5$/ml of digestive cells were placed in a 6-well culture plate with pre-treated cover slide in each well. cultured for 24h and then supernatant were discarded, added with medicine, continue to culture for 24hours ,meanwhile set blank control group,The cover slide were removed, fixed with acetone at 4°C for 10min and stained with SP method according to manual. The brown yellow cytoplasm indicated positive, and the expression intensity of Fas was inversely determined by the average gray value obtained with image analysis system ,which means the more is the average gray value, the less is the Fas level.

*Statistical method*

Experimental data were given as means±standarad deviations (SDs), compared between different groups by t-test with WindowsSPSS13.0 software.

## Results

*Changes of survival rates of tumor cells*

After the treatment of the cells at different temperature for 1h , when the concentration of paclitaxel was 1ug/ml, 10ug/ml, 50ug/ml and 100ug/ml, the cell growth was inhibited in a dose-dependent manner. There were significant differences between different groups (P<0.05) (Figure 1A). Similarly, when the concentration of carboplatin was 1μg/ml,5 μg/ml,10μg/ml and 100μg/ml, the cell growth was also suppressed between different groups (P<0.05) (Figure 1B). With the combination of 10μg/ml paclitaxel and 5 μg/ml carboplatin, the survival rate was significantly lower (P<0.01) (Figure 1C), compared with the respective use of 10μg/ml paclitaxel or 5μg/ml carboplatin, showing that the combined use of paclitaxel and carboplatin may have stronger inhibition effect than the single agent. More importantly, we found that cell growth was inhibited in a temperature-denpendent manner since the survival rate of OS732 cells were the lowest at 43°C and the highest at 37°C.

*Morphological changes of apoptosis of OS-732 cells*

Under the inverted phase contrast microscope, The normal OS-732 cells were attached to the dish, the cells

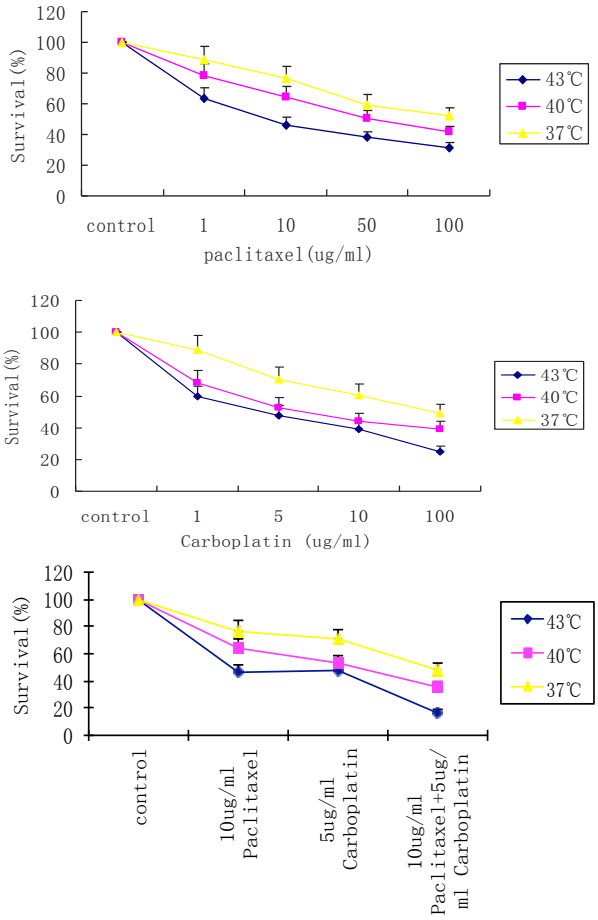


**Figure 1. Survival Rates OS732 at Different Temperature for 1h Measured by MTT.** (A) Survival rates with different concentrations of paclitaxel, (B) Survival rates with different concentration of carboplatin, (C) Survival rate with combination of 10μg /ml paclitaxel and 5μg /ml carboplatin

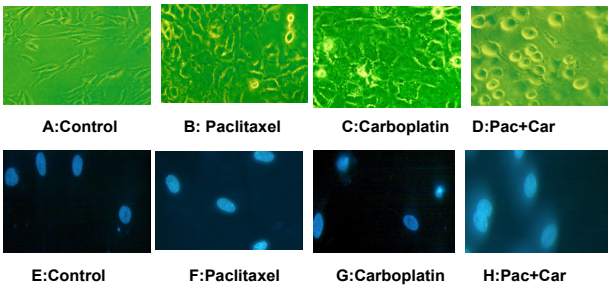


**Figure 2. Morphological Changes and Fluorescent Staining of OS732 Cells with Different Drugs at 43°C for 1h.** (A) Morphological appearance of OS732 under inverted phase contrast microscope ×400, (B) Morphological changes of OS732 treated with 10 μg /ml paclitaxel under inverted phase contrast microscope ×400, (C) Morphological changes of OS732 treated with 5μg /ml carboplatin under inverted phase contrast microscope ×400, (D) Morphological changes of OS732 treated with combination of 10μg /ml paclitaxel and 5μg /ml carboplatin under inverted phase contrast microscope ×400, (E) Fluorescent staining of OS732 cells under the fluorescence microscope ×400, (F) Fluorescent staining of OS732 cells treated with 10μg /ml paclitaxel under the fluorescence microscope ×400, (G) Fluorescent staining of OS732 cells treated with 5μg / ml carboplatin under the fluorescence microscope ×400, (H) Fluorescent staining of OS732 cells treated with 10μg /ml paclitaxel and 5μg /ml carboplatin under the fluorescence microscope ×400

were rhombus and angular, adhered-growing (Figure 2A). In the respective application of paclitaxel (10ug/ml) and carboplatin (5ug/ml), only part of the cells became small and round (Figure 2B, 2C), However, in the joint group, chromatin and cytoplasm condensed, many cells exfoliated and suspended in the culture solution (Figure 2D). Under fluorescence microscope, normal cells were evenly-distributed and lightly-stained (Figure 2E), When paclitaxel (10ug/ml) or carboplatin (5ug/ml) were used, only part of the cells showed condensed and flared fluorescence (Figure 2F, 2G). However, in the joint group, condensed and flared fluorescence may be observed, showing the presence of many apoptotic cells (Figure 2H).

*Comparison of apoptotic rates of OS-732 cells*

After the treatment of the cells at different temperature for 1h , when the concentration of paclitaxel was 1ug/ml, 10ug/ml, 50ug/ml and 100ug/ml, the apoptotic rate increased with a dose-dependent manner. There were significant differences between different groups ($P<0.05$) (Figure 3A). When the concentration of carboplatin was 1ug/ml, 5ug/ml, 10ug/ml and 100ug/ml, the apoptotic rate also increased with a dose-dependent manner. There were significant differences between different groups ($P<0.01$) (Figure 3B), with 10ug/ml paclitaxel and 5 ug/ml carboplatin, the apoptotic rate was significantly higher ($P<0.01$) (Figure 3C), compared with the respective use of 10ug/ml paclitaxel or 5ug/ml carboplatin, showing that the combined use of paclitaxel and carboplatin may have stronger apoptosis-inducing effect than the respective use.

*Fas expression of OS732 by Immunocytochemistry*

We observed only a small amount of brown particles in the cytoplasm of OS732 cells without drugs (Figure 4A).Deeper staining cytoplasm of OS732 cells with 10ug/

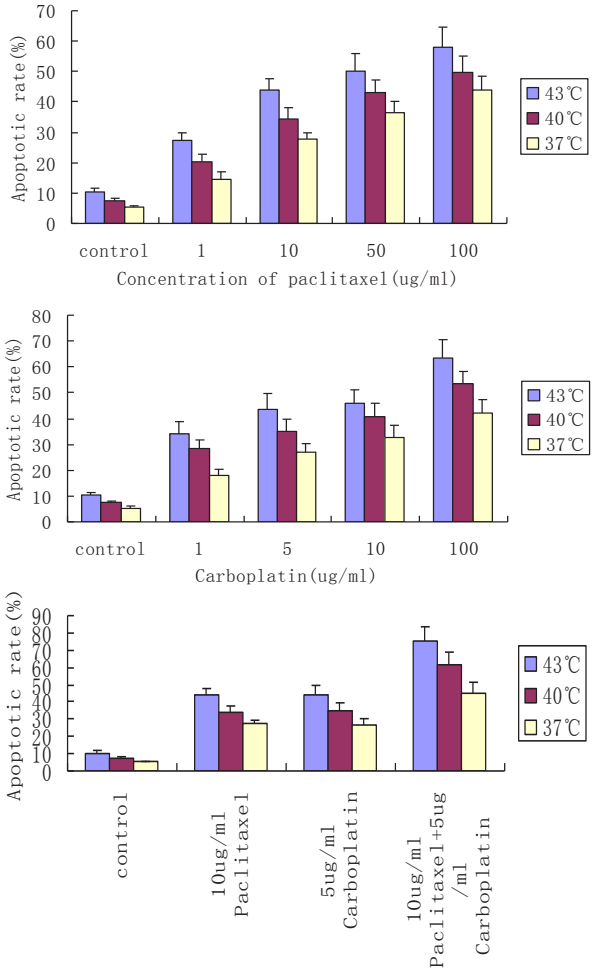


**Figure 3. Apoptotic Effect of OS732 Cells with Different Drugs at Different Temperature for 1h.** (A) Apoptotic rate of OS732 treated with different concentration of paclitaxel, (B) Apoptotic rate of OS732 treated with different concentration of carboplatin, (C) Apoptotic rate of OS732 treated with combination of 10μg /ml paclitaxel with 5μg /ml carboplatin

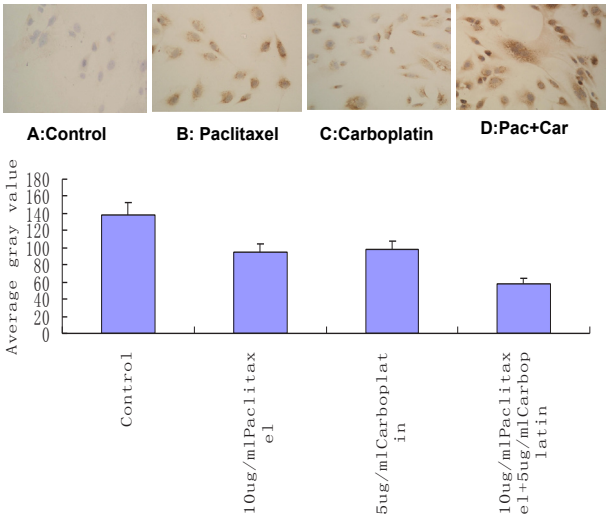


**Figure 4. Fas Expression of OS732 Cells with Different Drugs at 43°C for 1h.** (A) Immunocytochemistry ×400, (B) With 10 μg /ml paclitaxel by immunocytochemistry ×400, (C) With 5μg /ml carboplatin immunocytochemistry ×400, (D) Fas expression of OS732 treated with combination of 10μg /ml paclitaxel and 5μg /ml carboplatin by immunocytochemistry ×400, (E) Quantitative analysis of Fas level by comparing the average gray value of different groups with Meta Morph automatic image analyzer

ml paclitaxel (Figure 4B) or 5ug/ml carboplatin (Figure 4C); With the combination of 10ug/ml paclitaxel and 5ug/ml carboplatin, Deepest staining in cytoplasm, deformed, huge osteosarcoma cells is clearly visible, all Vision are covered with dye range (Figure 4D). we further measured the fas level quantitatively with Meta Morph automatic image analyzer by comparing average gray value which is Inversely proportional to the Fas expression (Figure 4E).

## Discussion

Thermochemotherapy is a comprehensive method that has been developed based on the hyperthermal therapy of malignant tumors. The isolated perfused chemotherapy of osteosarcoma hyperthermally may not only control the primary local tumor effectively, but also increase successful rates of limb salvage greatly ( Fan et al., 2003; Debes et al., 2005). Even though, the definite effect of thermotherapy with carboplatin has been extensively demonstrated in many tumor tissues (Bakshandeh-Bath et al., 2009; Fiorillo et al., 2009), there are only limited reports available in the field of osteosarcoma. It is well known that the optimal therapeutic condition clinically is 42-43°C for 1h, because this maximizes the tumor damage while preserving the surrounding normal tissue. We also found the experimental condition at 43°C was the perfect temperature for combination of paclitaxel and carboplatin to kill the OS732 cells lines in thermochemotherapy.

Currently, the thermotherapy-enhancing effect of carboplatin has reached a consensus (Bakshandeh-Bath et al., 2009; Fiorillo et al., 2009). As for paclitaxel, Many scholars hold positive views on the enhancement effect of paclitaxel in thermotherapy (Zoul et al., 2004; Michalakis et al., 2007; Liu et al., 2008), However, there are also contrary opinion, Mohamed reported that docetaxel cytotoxicity was enhanced by hyperthermia, however paclitaxel was not enhanced by hyperthermia (Mohamed et al., 2003).

We found after low dose of paclitaxel and carboplatin were used jointly at 43°C for 1h, the survival rate decreased sharply. The apoptotic rates of combination group obviously increased ($P<0.01$) mainly accomplished by inducing apoptosis (Figure 3C), which was similar to other research of different tumors (de Bree et al., 2006).

Nowadays there are two relatively consentaneous points on the anti-tumor mechanism of thermochemotherapy. Firstly, thermotherapy can change the membrane permeability of tumor cells so as to make drugs enter tumor cells easily, chemotherapy may be combined intraoperatively with hyperthermia, which enhances tissue penetration and cytotoxic activity of many drugs (de Bree et al., 2006). Secondly, thermotherapy can promote drugs to induce apoptosis of tumor cells. Many chemotherapeutic agents induce cellular apoptosis ultimately through different mechanisms which may be promoted by thermotherapy. The combined thermochemotherapy of paclitaxel and carboplatin may initiate the complicated apoptosis signal pathway producing the greatest killing-tumor effect. Our study showed that paclitaxol or carboplatin, to some extent,increased the Fas expression hyperthermally compared with the control group, however, combination of paclitaxol and carboplatin in the presence of hyperthermia greatly increased the fas expression of OS732 cells compared with the respective individual treatment (Figure 4).

Many research revealed that Fas-FADD signal played important role in the induction of aopotosis of tumor cell by carboplatin (Mishima et al., 2003; Li et al., 2007; Kim et al., 2009). Therefore our results are basicly consistent with above researches. As for the antitumor mechanism of paclitaxel,most researchers regard it as to induce cell accumulation in the G2/M-phase of the cell cycle (Drago-Ferrante et al., 2008). Some research revealed that Fas-FADD signal play important role in the aopotosis of tumor cell by paclitaxel (Stumm et al., 2004; Nuno et al., 2007). But there are also contray reports that Paclitaxel triggers cell death in H460 cells mainly via a currently unidentified caspase-independent mechanism (Huisman et al., 2005). Our results revealed that Paclitaxel can increase the fas-expression of OS732, however, we should not neglect that hyperthermia may also invole in the upregulation of Fas expression. As there are reports indicating that the hyperthermia can did influence the fas level (Yu et al., 2007; Wang et al., 2009), after all, we did find, in the presence of hyperthermia, combination of small dose of paclitaxel and carboplatin synergistically contributed to the up-regulation of Fas, which revealed us the possible mechamism of paclitaxel on thermochemotherapy of osteosarcoma. As the local thermal carboplatin infusion chemotherapy has been widely used clinically, we suppose, this kind of up-regulation fas mechanism will help to improve the sensitivity of thermo-chemotherapy with carboplatin, and maximize cytotoxity on primary tumor so as to prevent the recurrence after operation.

In conclusion, our results demonstrated that pacliataxel is capable of sensitizing the carboplatin on OS732 cell line in the presence of hyperthermia by up-regulation of Fas expression. Currently, paclitaxel has become the common drug for treatment of solid tumors other than osteosarcoma (Sirichaisutdhikorn et al., 2009; Zhou et al., 2009). However, we consider that paclitaxel used in thermochemotherapy of osteosarcoma would be a more ideal therapeutic method, As carboplatin and paclitaxel are both anti-tumor drugs with exact therapeutic effect, the toxicity and resistance may be easily caused after large-dose and long-term use, whereas the combined application of carboplatin and paclitaxel at small dose in the presence of hyperthermia will enhance the apoptosis-inducing effect so as to improve drugs sensitivity of osteosarcoma patients and minimize the cytotoxicity caused by clinical chemotherapy.

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