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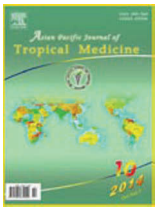

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# Influence of $As_2O_3$ combined with ginsenosides Rg3 on inhibition of lung cancer NCI-H1299 cells and on subsistence of nude mice bearing hepatoma

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ABSTRACT

**Objective:** To study the effect of arsenic trioxide ($As_2O_3$) combined with ginsenosides Rg3 on inhibiting the NCI-H1299 lung cancer cells and subsistence in nude mice bearing hepatoma. **Methods:** MTT method was used to measure the inhibition effect of $As_2O_3$ combined Rg3 on NCI-H1299 cells, and the proliferation inhibiting effect was observed via establishing the transplanted tumor model *in vitro*. A total of 40 tumor-bearing nude mice were randomly divided into normal saline group, $As_2O_3$, Rg3 and $As_2O_3$+Rg3 group. Transplantation tumor model of lung cancer in nude mice was constructed, followed by injection of certain concentrations of normal saline, $As_2O_3$, ginseng saponin Rg3 and $As_2O_3$+Rg3 every day. The survival duration and the tumors size of the mice were recorded and the Kaplan-Meier curve was made; microscopic observation of apoptosis of tumor cells *in vivo* was done using TUNEL staining. **Results:** After 72 h of injection, inhibition rate of tumor cell in normal saline group, $As_2O_3$ group, Rg3 group and $As_2O_3$+Rg3 group was (5.66±0.31)%, (65.58±4.75)%, (44.69±3.32)% and (82.67±5.43)%, respectively. Inhibition rate of tumor cell in $As_2O_3$ group, Rg3 group and $As_2O_3$+Rg3 group was significantly higher than that of normal saline group ($P$<0.01); inhibition rate of tumor cells of $As_2O_3$+Rg3 group was significantly higher than that of the two groups given $As_2O_3$ or Rg3 alone ($P$<0.01). The tumor volume of $As_2O_3$ group, Rg3 group and $As_2O_3$+Rg3 group shrank to (65.38±3.25)%, (77.68±3.43)% and (42.65±3.55)% of the original, tumor volume of saline group was 1.21 times of the original size ($P$<0.01); Median survival of saline group, Rg3 group, $As_2O_3$ group were significantly shorter than that of $As_2O_3$+Rg3 group ($P$<0.01); co-ordinated intervention ability of $As_2O_3$+Rg3 on NCI-H1299 cell was significantly higher than that of $As_2O_3$ or Rg3, separately. **Conclusions:** $As_2O_3$ combined with Rg3 can significantly inhibit proliferation of NCI-H1299 cells in lung cancer, prolong survival of tumor-bearing nude mice, and promote tumor cell apoptosis, and have significant effect on lung cancer treatment.

## 1. Introduction

Lung cancer is one of the most common malignant tumor in the world; its incidence and mortality are among the top of the global malignant tumors[1]. In recent years, the incidence of lung cancer in China shows a trend of sharp rise[2]. At present, the early treatment of lung cancer is mainly surgery, with chemotherapy as complementary. Studies have shown that[3], arsenic trioxide ($As_2O_3$) has the effect on differentiation and apoptosis of tumor cells. Researchers have conducted extensive researches on the anticancer mechanism of $As_2O_3$, and have confirmed significant inhibitory effect of $As_2O_3$ on the growth and proliferation of blood system tumor and solid tumor[4,5]. Ginsenosides Rg3 is a new type of antitumor drug extracted from ginseng. Studies have confirmed that[6-9], ginsenosides Rg3 could inhibit tumor development by inhibiting tumor angiogenesis, regulating apoptosis of tumor cell, controlling the proliferation and invasion and metastasis of tumor cell, and inhibiting multi-drug resistance. In the present study, the authors observed the inhibitory effect of $As_2O_3$ combined with Rg3 on NCI-H1299 cell by MTT method, and studied its treatment effect on the human lung cancer by constructing tumor model *in vitro*, so as to provide the theoretical basis of combination therapy of these two drugs for lung cancer treatment.

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## 2. Materials and methods

### 2.1. Experimental materials

Fetal bovine serum was purchased from Thermo Fisher Scientific Biological Chemicals (Beijing) Co., LTD.; $As_2O_3$ injection was bought from Beijing Double Heron Medicine; DMEM medium was purchased from Thermo Fisher Scientific Biological Chemicals (Beijing) Co., LTD.; MTT kit and TUNEL kit were purchased from Sigma Company. The rest of the reagents were of analytical grade. NCI-H1299 lung cancer cells were provided by Shanghai Institute of Cell Biology; and male nude mice were purchased from Chengdu Dashuo Experimental Animal Company.

### 2.2. Methods

#### 2.2.1. Cell culture

NCI-H1299 cells were added into DMEM medium containing 100 mg/L fetal bovine serum; the culture condition was 5% $CO_2$, saturated humidity, temperature of 37 ℃. The cells were digested with 2.5 mg/L of trypsin when cells convergence degree reached 0.8 to 0.9, and cells in logarithmic phase were used for experiment.

#### 2.2.2. Observation of the inhibitory effect of $As_2O_3$ combined with Rg3 on NCI- H1299 cell by MTT method

NCI-H1299 cells were cultivated in 96-well plates. When the cells completely adhered to the walls in the logarithmic phase, sterile filtered saline, $As_2O_3$ (1 mmol/mL), Rg3 (30 μg/mL), $As_2O_3$+Rg3 ($As_2O_3$ 1 mmol/mL, Rg3 30 μg/mL) were added, respectively. After cultivating the plates in $CO_2$ incubator at 37 ℃ for 24 h, 48 h and 72 h, 20 μL 5 mg/mL MTT was added in each orifice for another 4-hour incubation. The liquid was removed from the orifices, 200 μL DMSO was added in each orifice. The orifices were shaked at 37 ℃ away from light for 15 min, then the optical density was determined at 490 nm using microplate reader.

#### 2.2.3. Establishment of tumor model

The logarithmic phase of NCI-H1299 cells was digested with 0.125%, neutralized with culture medium containing serum, and centrifuged. After removing supernate, the cells were cultivated in 96-well plates pretreated with low melting point agarose and the plates were placed in $CO_2$ incubator at 37 ℃ for 7 d. $As_2O_3$ (1 mmol/mL), Rg3 (30 μg/mL), $As_2O_3$+Rg3 ($As_2O_3$ 1 mmol/mL, Rg3 30 μg/mL) were added for analyzing the volume change of tumor model, with saline as the negative control.

#### 2.2.4. Establishing transplanted tumor model of lung cancer and observing the inhibitory effect of $As_2O_3$ combined with Rg3 on lung cancer

Male nude mice of 4-6 weeks, 20-25 g were selected and fed in clean grade experimental centre under light cycle of 12/12 h. Relative humidity was 70% and temperature was 25 ℃. NCI-H1299 cells were digested by trypsin. After centrifugation, cells were suspended in DMEM medium and the concentration was adjusted to $5\times10^7$/mL. NCI-H1299 cell suspensions (0.1 mL) were inoculated into each nude mouse subcutaneously. The model was proved successful once tumor block was visible one to two weeks later. After optimization, 40 tumor-burdened nude mice were randomly divided into four gourps with 10 in each: the normal saline group (placebo therapy with saline intravenous injection at rat tail); the $As_2O_3$ group (3 mg/kg $As_2O_3$ injection); the Rg3 group (10 mg/kg Rg3 injection) and the $As_2O_3$+Rg3 group ($As_2O_3$ 3 mg/kg+Rg3 10 mg/kg at rat tail). From the 7th day after successfully modeling, the injection was adopted every 3 d in each group respectively. Weight of nude mice in each group after injection was measured until the natural death, and the survival life span of each group was recorded.

### 2.3. TUNEL staining

After the natural death, tumor tissue was extracted for conventional preparation of biopsies; the tissue slice was fully dewaxed with xylene, hydrated with gradient ethanol and incubated for 10 to 30 min with proteinase k. Then TUNEL reaction drops were added, incubated at 37 ℃ for 1 h, followed by color development for 10 min with DAB, fully rinsed with PBS and sealed with POD. The apoptosis of tumor cells microscopically was observed; apoptosis of tumor cells was shown in brown.

### 2.4. Statistical methods

SPSS21.0 statistical software was used for data analysis. Measurement data were calculated as mean±SD, and compared between groups using ANOVA. $P<0.05$ was regarded as statistically significant.

## 3. Results

### 3.1. Comparison of proliferation inhibition on NCI-H1299 cells

MTT experiment showed that with time increased, $As_2O_3$ and Rg3 both inhibited proliferation of NCI-H1299 tumor cells and this effect was time dependent. Co-ordinated intervention ability of $As_2O_3$+Rg3 on NCI-H1299 cell was significantly higher than that of $As_2O_3$ or Rg3 separately. After 72 h of injection, inhibition rates of tumor cell in normal saline group, $As_2O3$ group, Rg3 group and $As_2O_3$+Rg3 group were (5.66±0.31)%, (65.58±4.75)%, (44.69±3.32)% and (82.67±5.43)%, respectively. Inhibition rates of tumor cell in $As_2O_3$ group, Rg3 group and $As_2O_3$+Rg3 group were significantly higher than that of normal saline group ($P<0.01$); the results are shown in Figure 1.

### 3.2. Comparison of inhibition on NCI-H1299 tumor growth

Experimental results showed that both $As_2O_3$ and Rg3 could inhibit tumor growth. After 7 d treatment, the tumor volume of $As_2O_3$ group, Rg3 group and $As_2O_3$+Rg3 group shrank to (65.3±3.25)%, (77.68±3.43)% and (42.65±3.55)% of the original size. Tumor volume of saline group was 1.21 times of the original size ($P<0.01$) (Figure 2).

### 3.3. Weight change of mice in the process of drug delivery

There were no obvious changes in weight in mice of each

group, as shown in Figure 3.

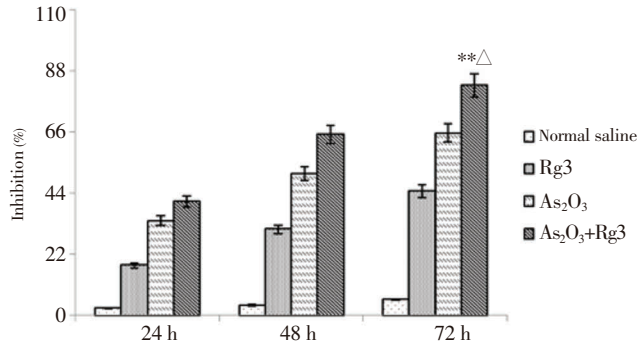


**Figure 1.** Influence of different drugs on cell proliferation of lung cancer cells.
**: $P<0.05$, difference is statistically significant compared with $As_2O_3$ or Rg3 alone; △: $P<0.05$, difference is statistically significant compared with 24 h and 48 h $As_2O_3$+Rg3.

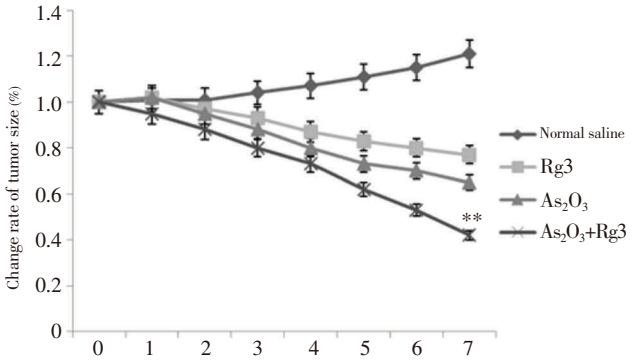


**Figure 2.** Volume change of the tumors after 7 d treatment.
**: $P<0.01$, difference is statistically significant compared with $As_2O_3$ and Rg3 groups, respectively.

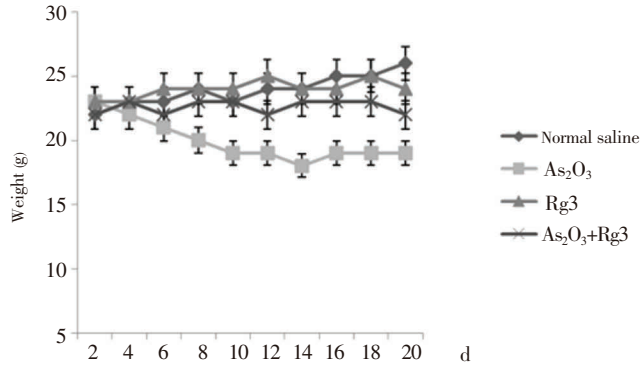


**Figure 3.** Weight change of mice in the process of drug delivery.

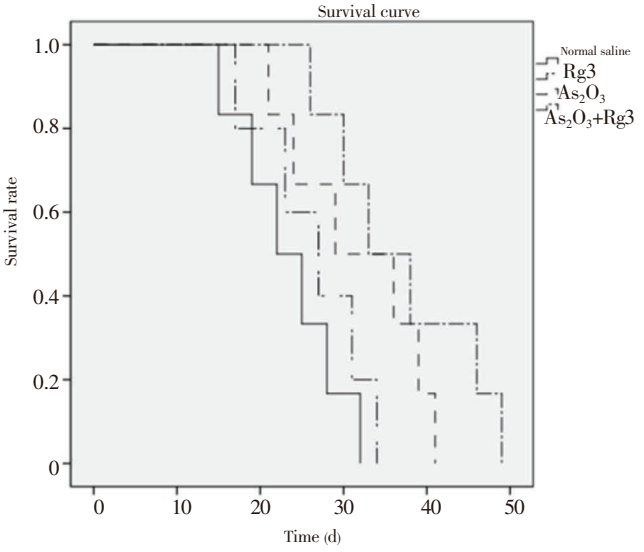


**Figure 4.** Kaplan-Meier curve of mice in different groups.

### 3.4. Median survival between groups

Median survival of saline group, Rg3 group, $As_2O_3$ group and $As_2O_3$+Rg3 group were 21, 26, 31 and 41 d; the injection of $As_2O_3$+Rg3 can significantly prolong the median survival of tumor-bearing nude mice, as shown in Figure 4.

### 3.5. TUNEL dyeing

Tumor cells in apoptosis were shown as brown (Figure 5). There were most tumor cells induced apoptosis in $As_2O_3$+Rg3 group, followed by $As_2O_3$ group, Rg3 group and saline group.

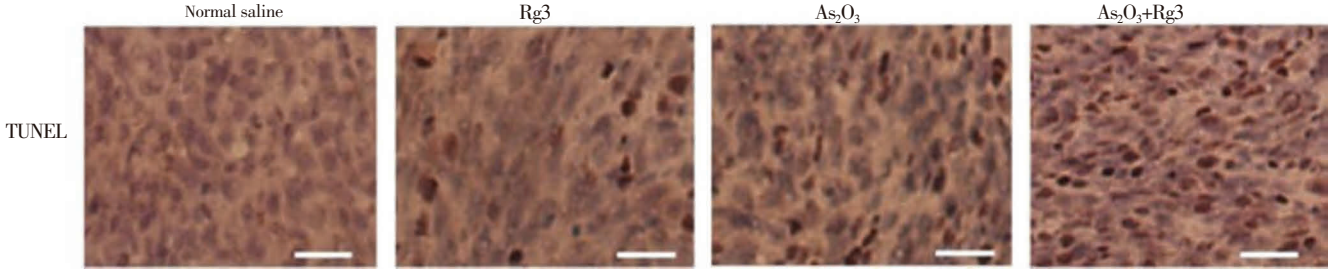


**Figure 5.** TUNEL dyeing results of tumor cells of differnt groups.

## 4. Discussion

Lung cancer is a common malignant tumor. Its incidence and mortality tops among malignant tumors in the world, with rising trend year by year[2]. Studies have shown that[10-13], $As_2O_3$ can induce differentiation and apoptosis of tumor cells with cytotoxic effect, and its curative effect in treatment of leukemia and other substantive tumor has been confirmed clinically. Other studies have suggested[14-17], $As_2O_3$ can inhibit proliferation of tumor cells, induce mature tumor cells, inhibit tumor angiogenesis, and can affect immune function of tumor-burdened organism. Rg3 is a kind of traditional Chinese medicine extract from ginsenosides, and its antitumor activity has been widely used in the treatment of lung cancer, colon cancer and melanoma tumors[18-22]. The author observed inhibition effect of $As_2O_3$ with Rg3 ginsenosides on treatment of lung cancer NCI-H1299 cell by MTT method, built *in vitro* tumor model, observed $As_2O_3$ joint ginseng saponin Rg3 for lung cancer treatment effect, and explored the combination therapy for lung cancer by establishing tumor model, so as to

provide theoretical basis for clinical applications of the two drugs.

Proliferation inhibition in $As_2O_3$+Rg3 group on NCI-H1299 cells was significantly stronger than that in other three groups ($P$<0.01), indicating that $As_2O_3$ joint Rg3 ginsenosides can effectively inhibit lung cancer NCI-H1299 cells' proliferation, stopping lung disease process. A study showed[23], in some solid tumors because of the compact tumor tissue growth, high internal pressure and less vascular distribution, antitumor drug could hardly be delivered into tumor tissues[24,25]. Therefore, the authors established the *in vitro* tumor model to evaluate drug delivery ability to lung cancer. After 7 d, tumor volume in $As_2O_3$+Rg3 group shrank the most significantly, followed by (65.38±3.25)% in $As_2O_3$ group and (77.68±3.43)% in Rg3 group respectively. Tumor volume in saline group increased, suggesting that $As_2O_3$ combined with Rg3 can inhibit the growth of tumor more significantly than applying the two drugs separately. From the treatment results of the tumor-bearing nude mice, $As_2O_3$+Rg3 group showed significantly higher median surial than that of the other three groups, suggesting that $As_2O_3$ combined with Rg3 can enhance survival of tumor-bearing nude mice. In addtion, TUNEL staining results showed that $As_2O_3$+Rg3 group had the highest apoptosis rate of tumor cells, confirming the apoptosis promoting effect of $As_2O_3$+Rg3 on tumor cells.

It can be concluded that $As_2O_3$ combined with Rg3 can significantly inhibit the proliferation of NCI-H1299 cells in lung cancer, prolong survival of tumor-bearing nude mice, and promote tumor cell apoptosis, and have significant effect on lung cancer treatment.

## Conflict of interest statement

We declare that we have no conflict of interest.

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