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Preparation and evaluation of As_2O_3 nanoparticles for treatment of human liver cancer cells in vitro

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Abstract: This paper studies the preparation method of As_2O_3 nanoparticles and their antitumor effect on human liver cancer cells. As_2O_3 nanoparticles were prepared by sol-gel method. As_2O_3 nanoparticles were characterized by transmission electron microscope (TEM), energy dispersive spectrometer (EDS) and computer color magic image analysis system (CMIAS). A methyl thiazolyl tetrazolium (MTT) assay and a flow cytometry (FCM) assay were performed to examine the antitumor effect of As_2O_3 nanoparticles at various concentrations (1, 2, 5, 10 μmol/L). We also compared the antitumor effect of As_2O_3 nanoparticles with that of As_2O_3 solution. The average diameters of two kinds of As_2O_3 nanoparticles prepared were about 80 nm and 40 nm. It was identified that the prepared nanoparticles were As_2O_3 and there were no other components by EDS. After 48 h of treatment with As_2O_3 nanoparticles, the survival ratio of cells was significantly lower than that of the As_2O_3 solution with the same concentration ($P < 0.05$). Experimental results demonstrate that by sol-gel method As_2O_3 can be prepared into nanoparticles. As_2O_3 nanoparticles can produce a better cytotoxic effect on tumor cells than the As_2O_3 solution.

Key words: nanotechnology; arsenic trioxide; liver cancer

Nanotechnology is a new research field that has developed rapidly in recent years, producing an enormous influence on many other research fields. A complete list of the potential applications of nanotechnology is too vast and diverse to discuss in detail, but undoubtedly one of the greatest contributions of nanotechnology will be the development of new and effective medical treatments. The application of nanotechnology to medicine is called nanomedicine; it has many advantages over normal medicine such as improvement of solvency and increasing of targeting ability[1].

Arsenic trioxide (As_2O_3) has been adopted from traditional Chinese medicine and used successfully to treat refractory or relapsed acute promyelocytic leukemia (APL)[2,3]. Zhang, et al[2]. reported a satisfactory result by using As_2O_3 for the treatment of early APL. In recent years, some researchers found that As_2O_3 was not only effective in leukemia treatment, but also effective in inhibiting several animal and human solid cancer cell lines such as cervical cancer[4], esophageal cancer[5], gastric cancer[6], nasopharyngeal cancer[7], liver cancer[8], prostate cancer[9], ovarian cancer[9] and breast cancer[10].

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1 Materials and Methods

1.1 Reagents, instruments and cell line

1) Reagents As_2O_3 (Sigma Co.), RPMI1640 (GIBCOL-BRL), new-born calf serum (Sijiqing Biotechnology Co., Hangzhou), HEPES (AMRESCO), Trypsin (AMRESCO), MTT (AMRESCO).

2) Instruments Transmission electron microscope (TEM, H-600, Hitachi, Japan), scanning electron microscope (SEM, JEOL JSM-6360LV, Japan), energy dispersive spectrometer (EDS, Thermo NORAN Vantage, USA), computer color magic image analysis system (CMIAS, 98A, Beijing University of Aeronautics and Astronautics), microplate reader (Multiskan MK3-353, USA), flow cytometry (FCM, Vantage SE, BD Company, USA).

3) Cell line SMMC-7721 cells (human liver cancer cell lines) provided by the Institute of Biochemistry and Cell Biology, Shanghai Institute of Biological Sciences, Chinese Academy of Sciences.

1.2 Methods

1.2.1 Preparation of As_2O_3 nanoparticles

As_2O_3 nanoparticles were prepared using sol-gel method[11]. Sol-gel method is a common method used to prepare nanoparticles. The formulae are as follows:

$M(OR)_n + xH_2O \rightarrow M(OH)_x(OR)_{n-x} + xROH$; $M(OH)_x(OR)_{n-x} \rightarrow (x - n/2)H_2O + (n - x)ROH + M_xO_{n/2}$ (M = metal element, $R = C_yH_{2y+1}$).

1.2.2 Determination of As_2O_3 nanoparticles characters

As_2O_3 nanoparticles were taken on cuprum grid with membrane and observed under TEM. TEM pictures were taken in H-600 TEM. The average diameters of As_2O_3 nanoparticles were measured by CMIAS. The composition of As_2O_3 nanoparticles was analyzed by SEM and EDS.

1.2.3 Methyl thiazolyl tetrazolium (MTT) assay

SMMC-7721 cells were cultured in an RPMI1640 supplement with 10% heat-inactivated calf serum, penicillin (100 unit/mL) and streptomycin (100 mg/mL) and grown in the presence of 5% CO_2 in air at 37 ℃. The cells were seeded in a 96-well plate with 6 000 cells per well and treated with As_2O_3 and As_2O_3 nanoparticles at various concentrations (1, 2, 5, 10 μmol/L) for 1, 2, 3 and 4 d. Then 20 μL MTT (5 g/L) was added to the cells in every well and incubated for 4 h at 37 ℃. Culture media was replaced by 150 μL dimethyl sultfoxide and vibrated for 10 min. Then optical density (OD) values were measured at a wavelength of 493 nm. The cell survival ratio was counted with the following formula: survival rate(%) = (OD of the treated group)/(OD of the untreated group) × 100%.

1.2.4 Flow cytometric analysis of apoptosis

Cell apoptosis and cell cycle were identified and quantified by FCM. 10^6 cells of the control group and experimental group were detached with trypsin/EDTA and collected and washed twice with 0.1 mol/L PBS (pH = 7.2 to 7.4), resuspended and fixed in 70% ethanol at 4 ℃ overnight. Cells were centrifuged, resuspended in propidium iodide (20 μg/mL, 0.25 mg/mL RNase A) at 37 ℃ for 30 min in the dark. After staining, cells were immediately evaluated by flow cytometry and the data were analyzed by Lysis Ⅱ software.

1.2.5 Statistical analysis

Values were expressed as mean ± SD. The data were analyzed with SAS 10.0. The significance was analyzed by using a *t*-test. $P < 0.05$ was the criterion for statistical significance.

2 Results

2.1 Characters of As_2O_3 nanoparticles

Observed by TEM, powders of As_2O_3 were square or polygonal or anomalistic crystal with high electronic density. Average diameters of As_2O_3 powder were about 5 μm(see Fig. 1(a)). As_2O_3 nanoparticles were round or elliptical, well dispersive and about 80 nm (see Fig. 1(b)) and 40 nm (see Fig. 1(c)) in diameter. Average diameter of As_2O_3 nanoparticles were (80 ± 0.01) nm and (40 ± 0.01) nm measured by CMIAS(see Tabs. 1 and 2). It was identified that these nanoparticles were As_2O_3 by EDS and quality fraction of As was 8.71%. There were no other components (see Fig. 2).

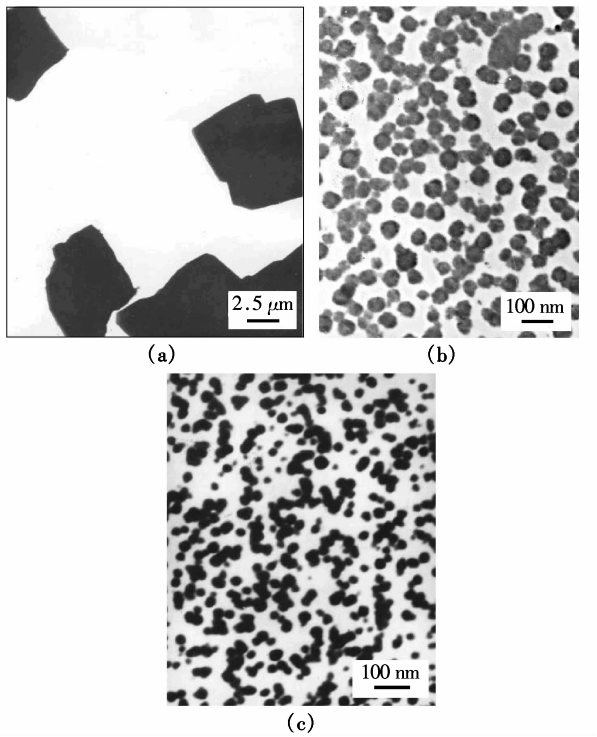

Fig. 1 Shape of As_2O_3 powder and As_2O_3 nanoparticles

Tab. 1 Plane parameters of As_2O_3 nanoparticles 1(total 600)

Classification	Area/μm^2	Perimeter/μm	Long pathway/μm	Short pathway/μm	Average diameter/μm
Summation	3.41	167.06	55.88	45.05	50.52
Mean	0.01	0.28	0.09	0.08	0.08
Standard deviation	0.00	0.04	0.01	0.01	0.01
Coefficient of variation	0.282	0.152	0.149	0.153	0.141

Tab. 2 Plane parameters of As_2O_3 nanoparticles 2(total 610)

Classification	Area/μm^2	Perimeter/μm	Long pathway/μm	Short pathway/μm	Average diameter/μm
Summation	0.96	87.32	30.28	22.30	26.84
Mean	0.00	0.14	0.05	0.04	0.04
Standard deviation	0.00	0.04	0.01	0.01	0.01
Coefficient of variation	0.510	0.267	0.260	0.237	0.255

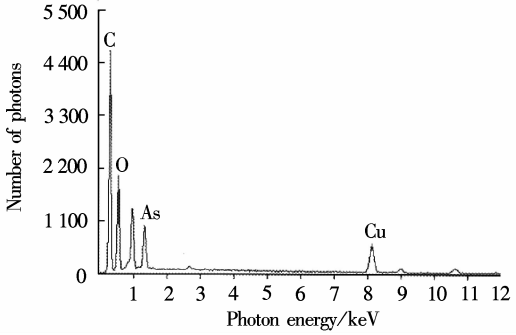

Fig. 2 EDS of As_2O_3 nanoparticles

2.2 As_2O_3 nanoparticles-inducing apoptosis in human hepatocellular carcinoma cells

SMMC-7721 cells in the control group exhibited a normal shape, clear edge, numerous ones and no fragments of cells (see Fig. 3(a)). SMMC-7721 cells decreased, unshaped and edge-indistinct after treatment with As_2O_3 solution, at the same time, the number of necrotic cells and cellular fragments increased (see Fig. 3(b)). With the same concentration of As_2O_3 nanoparticles the changes of SMMC-7721 cells became more obvious (see Fig. 3(c)). There was no distinct difference between two nanoparticles. As shown in Fig. 4, the survival ratio of cells treated by As_2O_3 nanoparticles was significantly lower than cells treated with As_2O_3 solution with the same concentration and incubation time. Results of FCM confirmed the results mentioned above (see Fig. 5). In Fig. 5, group 1 is control group; group 2 is 5 μmol/L As_2O_3 solution group and As_2O_3 nanoparticles group; group 3 is 10 μmol/L As_2O_3 solution group and As_2O_3 nanoparticles group. As_2O_3 nanoparticles have stronger ability to induce apoptosis than As_2O_3 solution.

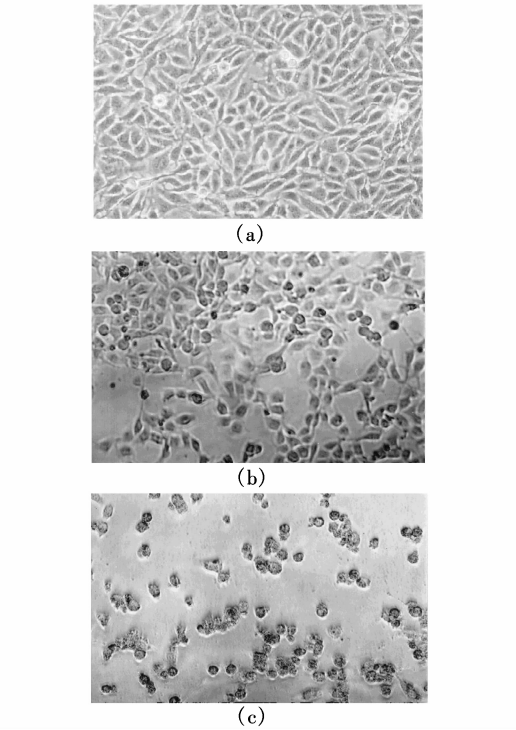

Fig. 3 Antitumor effect of As_2O_3 and As_2O_3 nanoparticles on SMMC-7721 cells after 48 h of treatment with 10 μmol/L. (a) Control group; (b) As_2O_3 solution group; (c) As_2O_3 nanoparticles group

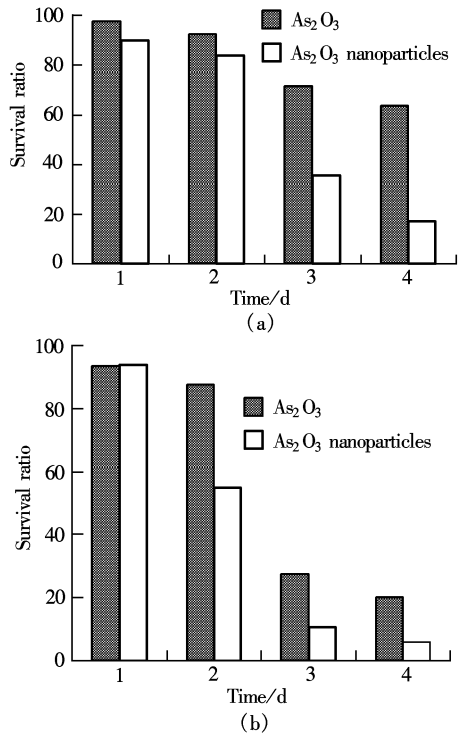

Fig. 4 Comparison of effect on survival ratio of SMMC-7721 cells between As_2O_3 nanoparticles and As_2O_3 solution. (a) Treated with 5 μmol/L As_2O_3 nanoparticles and As_2O_3 solution; (b) Treated with 10 μmol/L As_2O_3 nanoparticles and As_2O_3 solution

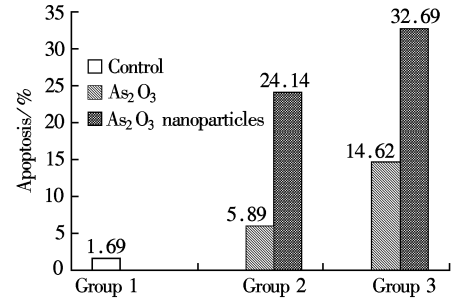

Fig. 5 Apoptosis in control cells and treated cells

3 Discussion

Hepatocellular carcinoma is one of the most common malignant tumors in China[12, 13], and its incidence has apparently increased in recent years. Surgical resection has been recognized as the most effective method for the treatment of hepatocellular carcinoma. As_2O_3, a traditional Chinese medicine, is generating

much interest as a novel anticancer therapeutic and is currently being tested both in the clinic and laboratory against various malignancies including hepatocellular carcinoma. Arsenic acts on cells through a variety of mechanisms, influencing numerous signal transduction pathways and resulting in a vast range of cellular effects that include apoptosis induction, growth inhibition, promotion of differentiation, and angiogenesis inhibition[14]. But its application to solid carcinomas is limited because of its poor dissolution. When treated with As_2O_3 patients will suffer from some acute and chronic side effects such as gastrointestinal response, some of which are often severe or fatal[15, 16]. Moreover it has been generally considered as an extremely effective environmental cocarcinogen for some human malignancies, especially for skin and lung cancer[17]. Investigations of epidemiology indicated that incidence of some tumors was associated with high levels of arsenic in drinking water[18]. So it is important to enhance the curative effect and reduce toxicity by changing the form of As_2O_3.

When normal particles become nanoparticles (1 to 100 nm), they will have some new characteristics especially a tremendous increase in surface area. For example, a particle of 10 nm has 90 m^2/g surface area, however, 5 nm particle has 180 m^2/g. Large surface area makes the number of exterior atoms increase sharply, and makes particles have high chemical energy. So there is a size-dependent effect of nanomedicine particles. Numerous studies have also demonstrated that the particle size of the nanosphere is crucial for uptake and transport across the gastrointestinal tract mucosal barrier and the expected pharmacodynamic activity[19]. It is easier for nanoparticles to enter the tumor cells to kill tumor cells than it is for normal particles[20]. Furthermore, side effects and foreign body irritation can be avoided. The modernization of traditional Chinese medicine is an important field with brilliant prospects; its nano-scale research has become one of the special areas of interest in this field. Zhang, et al. [21] have reported using microspheres as carriers combined with As_2O_3 to treat solid cancer. Ying, et al. [22] studied the effect of another arsenic-containing compound, realgar (As_2S_2) on ECV-304 cells. They reported general particles could hardly effect cells, however, realgar nanoparticles of diameters 100 nm and 150 nm could inhibit remarkably cell viability by apoptosis. The literature about preparing As_2O_3 into nanoparticles is currently insufficient.

As_2O_3 is a mineral Chinese medicine and cannot dissolve in water. In our study, As_2O_3 powders were prepared into nanoparticles and their average diameters were 80 nm and 40 nm. We can see that As_2O_3 nanoparticles have good dispersity and little reunition under TEM. The increase of pharmaceutical effect of As_2O_3 may be attributed to its tremendous surface area and chemical energy. However, the technique to control the diameter of nanoparticles was not well controlled. We have not prepared bigger or smaller As_2O_3 nanoparticles. Experimental results demonstrate that there is not a distinct difference between the two types of nanoparticles based on its antitumor effect. The possible reason for this problem is that the distance in diameters between two nanoparticles is not big enough.

In conclusion, by sol-gel method As_2O_3 can be prepared into nanoparticles. The As_2O_3 nanoparticles can produce a better cytotoxic effect on tumor cells than the As_2O_3 solution. This study may develop a new application of As_2O_3 for treatment of solid tumors. But the technique has a long way to go before being applied to clinical treatment; many problems remain to be solved.

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As_2O_3 纳米粒的制备、表征和体外治疗人肝癌细胞的研究

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摘要: 研究了 As_2O_3 纳米粒的制备方法、表征及其抗肝癌细胞的作用. 采用溶胶−凝胶法制备 As_2O_3 纳米粒, 并用透射电镜、能谱仪和图像分析仪等方法对其进行表征及特性检测. 通过 MTT 法和流式细胞仪法研究了不同浓度 As_2O_3 纳米粒对人肝癌细胞株的影响, 并与传统的 As_2O_3 溶液进行了比较. 实验中制备了 2 种粒径大小的 As_2O_3 纳米粒子, 其平均直径分别为 80 nm 和 40 nm, 通过能谱仪的测试证实所制备的纳米粒为 As_2O_3, 且无其他成分. 体外细胞实验发现, As_2O_3 纳米粒处理细胞 48 h后, 人肝癌细胞的存活率明显低于同浓度的 As_2O_3 溶液处理组($P < 0.05$). 研究结果显示, 通过溶胶−凝胶法可将 As_2O_3 制备成纳米粒子; 体外细胞实验表明, 与传统的 As_2O_3 溶液相比, As_2O_3 纳米粒子可对肿瘤细胞产生更强细胞毒作用.

关键词: 纳米技术; As_2O_3; 肝癌

中图分类号: R73-36