

Inhibitory Effect of Nano-HA Particles on Human U87 Glioblastoma Cells Viability

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Abstract: To evaluate the effects of Hydroxyapatite nano-particles (nano-HA) with different morphologies on human U87 glioblastoma cell line (U87), three kinds of nano-HA were designed and synthesized, and a co-culture system containing nano-HA and U87 cells was established. Polyethylene glycol (PEG) was also added to the HA medium system for the samples stabilization. After determining the stabilization and high dispersed nanometer size of nano-HA in the medium system using Transmission electron microscope (TEM) and dynamic light scattering (DLS), the effects of nano-HA on GBM cells were assessed with an MTT method. Results showed that nano-HA could effectively inhibit U87 cells proliferation. Compared with other nano-HA, the needle-like nano-HA were more powerful in inhibiting U87 cells proliferation. Toll-like receptor 4 (TLR4) induced by different nano-HA may be one reason for their different bioactivity.

Key words: hydroxyapatite; glioblastoma; Toll-like receptor 4; polyethylene glycol

Glioblastoma multiforme (GBM) is a lethal brain tumor that is a leading cause of solid tumor death in people under 20, and accounts for 25% of all primary brain tumors and 50% of gliomas in adults^[1]. Despite aggressive surgical resection and concurrent radiochemotherapy regimens, the prognosis for glioblastoma multiforme patients remains extremely dismal with a 2-year survival rate below 27%^[2]. Thus, great efforts have been made in the last decade to find out new therapies such as gene treatment and immune treatment. In these studies, nano-HA as a widely applied biomaterial in medicine has aroused intensive interest due to its biocompatibility and inhabitation of tumor cells^[3].

Hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HA) characterized by osteoconductive, non-toxic, non-immunogenic properties can induce bone formation on its surface^[4]. It was confirmed that nano-HA can inhibit the proliferation of various cancer cells^[5-7]. Recently, numerous evidences have indicated that the effect of nano-HA was related to the particles size. Shi, *et al*^[8] revealed that proliferation and apoptosis of human osteoblast-like MG-63 cells were affected by the HA particles size. Furthermore, Cai, *et al*^[9] showed that the particle size played a key role in the regulation of cell function. NP20 HA promoted the proliferation of bone marrow mesenchymal stem cells (MSCs), whereas, nano-HA in the same size were the best retardant for osteosarcoma cell growth. In

addition, the morphology of nano-HA might also be critical. However, the reported effects of morphology of nano particles on cancer cells were still controversial. Li, *et al*^[10] demonstrated that particle nanoscale rather than particle morphology was related to the inhibition of malignant melanoma cells proliferation. Nevertheless, this theory is not convincing enough in that nano-HA exist in various shapes besides rod-like and ellipse-like ones. So, the two following questions were studied on how nano-HA particles size affects glioblastoma cells and whether the morphology of nano-HA affects the proliferation of glioma cells. To investigate the biological effect of nano-HA on glioma cells, nano-HA with different shapes and sizes were synthesized. Sphere-like, rod-like and needle-like particles were diffused in medium respectively and their effects were assessed by MTT assay.

To investigate the mechanism of nano-HA inhibition, Toll-like Receptor 4 (TLR4) expression in U87 cells was also detected by RT-PCR assay. TLR4 belongs to a large innate receptor repertoire family (TLRs, TLR1-TLR11), which have been discovered to recognize conserved molecular patterns of microbial origin not expressed in host, and mediate the production of cytokines necessary for the development of effective immunity. The significance of TLR4 expression in tumor cells has been explored in hepatocellular carcinoma, epithelial ovarian cancer, breast cancer, lung cancer and neuro-

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blastoma^[11]. It has been reported that the TLR4 was intimately related to the HA particles' bioactivity, so we supposed that the TLR4 expression might be involved in the nano-HA particles effects on U87 cells.

1 Experimental

1.1 Preparation of HA nano-particles

Sphere-like nano-HA with an average diameter of 20nm (np20) and 80nm (np80) were fabricated using Hexadecyl (cetyl) trimethyl ammonium bromide (CTAB) to control particle size^[9]. 5.0mmol/L CaCl₂ (60mL) was dropped into 240mL solutions containing 1.25mmol/L Na₂HPO₄ and 6×10^{-4} , 12×10^{-4} mol/L CTAB respectively in magnetically stirred vessels at 20°C. The pH of the solutions was maintained at 9.5. The suspension was then stirred at 20°C for another 24h. The precipitates were separated by centrifugation and filtration, and then were washed completely with ethanol to remove the residual CTAB molecules.

Rod-like nano-HA (80 ± 10) nm in length and (20 ± 10) nm in width were synthesized^[12]: Ca(OH)₂ (3.71g) was dispersed in 100mL of deionized water, and stirred for 5h. 85% H₃PO₄ was diluted and added to Ca(OH)₂ suspension dropwise while stirring. The ratio of Ca/P was kept at 1.67. Thus, a white HA gel was formed. 20mL of the gel without aging was to form a suspension and then NH₄OH was added to obtain pH value of 11. The suspension was transferred to an autoclave and treated hydrothermally at 300°C for 2h.

Needle-like nano-HA were electro-deposited on the titanium substrate as described in reference [13]. Commercially available titanium (TA 15) plates 20mm × 20mm × 2mm in size were used as the cathode, and a platinum foil 20mm × 20mm × 1mm in size as a counter electrode. Specimens were electrodeposited at 10mA/cm². The particles were electrodeposited and the shapes were regulated by deposited durations of 5min^[14].

1.2 XRD, TEM, FTIR, and DLS characterization

The phases of the prepared powders were analyzed by X-ray diffraction (XRD) in a Huber D8211 diffractometer, with CuKα radiation, $2\theta = 20^\circ - 60^\circ$, at a scanning rate of 5°/min, a voltage of 40kV, and a current of 30mA. The structure and crystal size in medium system were evaluated by TEM (JEM-1200EX), and the size distribution was measured by Marlvern Nano Zetasizer (25 degree). The shpere-like samples were studied by Fourier transform infrared spectroscopy (FTIR,

Nicolet-380, America, 400 – 4000cm⁻¹).

1.3 Cell culture

Human U87 glioblastoma cell line was purchased from the American Type Culture Collection (ATCC) and maintained in a standard tissue culture incubator with 5% CO₂ in air and 100% relative humidity at 37°C in the lab. In brief, the cells were grown in Dulbecco's modified essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin G, and 100μg/mL streptomycin, and the medium was changed twice a week. U87 cells grew as monolayer in flasks. When the cells covered more than 90% area of the bottom, they were detached from the cell culture flask by washing with phosphate-buffered saline (PBS) and brief incubation with trypsin (0.5g/L)/EDTA (0.2g/L), then passaged in the ration of 1:3.

1.4 Samples preparation and challenge *in vitro*

Different autoclaving nano-HA particles were dispersed in DMEM. To prevent nano-particles agglomeration, polyethylene glycol (PEG-200, Sinopharm Chemical Reagent, Beijing, China) was used as a perfect dispersant^[15]. PEG-200 was added into the mixture at 1.5mg/mL, and dispersed using ultrasonication for 5min. The frequency of the ultrasound was fixed to be 24kHz at the power of 400W. The dispersions were sonicated in an ice cold-water bath to prevent over-heating.

1×10^4 U87 cells were seeded into round-bottomed 96-well microculture plates (Corning, United States) in a volume of 100μL medium per well, and incubated at 37°C for 24h for cells adherence. Then the medium was replaced by 100μL of DMEM containing four types of nano-HA, at different concentrations from 25μg/mL to 100μg/ml for 48h. Every concentration has tripled. Cells were incubated with or without PEG-200, which was dissolved in medium at 1.5mg/mL as a control.

For RT-PCR analysis, U87 cells were co-cultured with different nano-HA at IC50 (50% inhibitory concentration) for 30min, and PBS was used as blank control.

1.5 MTT (Methyl tetrazolium cytotoxicity) assay

The effect of nano-HA on glioma cells was determined using MTT assay. Briefly, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) (Sigma, United States) was dissolved in phosphate buffered saline (PBS, pH 7.4) at 5mg/mL and sterilized through a micropore filter. Wells of 96-well plates were filled with 20μL MTT at different time points, and incubated at 37°C for 4h, and then 100μL dimethyl sulfoxide

(DMSO, sigma-Aldrich) was added to each well with intensive mixing to dissolve the blue-violet crystals. Plates were read with an ELISA reader (TECAN SUNRISE) at 490nm within 1h after DMSO was added. The results were represented by percentage viability compared to the untreated controls. The survival rate could be calculated according to the formula $[A]_t/[A]_i$, where $[A]_i$ is the optical absorption of untreated cells and $[A]_t$ is the optical absorption of treated cells. The inhibitory rate (*IR*) was calculated by the following formula: $IR = (1 - \text{average OD490 of treated group} / \text{average OD490 of the control}) \times 100\%$.

1.6 RT-PCR assay

RT-PCR was employed to detect TLR-4 mRNA expression levels in U87 cells. Total RNA was extracted from U87 cells using Trizol reagent (Invitrogen, United States). 1μg RNA was used as template for reverse transcription. cDNA was synthesized with First-Strand Synthesis System for RT-PCR Kit(Invitrogen, United States) according to the manufacturer’s instruction. RT-PCR analyses were performed using the following primer sets for TLR4: forward primer 5'-TCCTTACCCAGTCCTCATC-3', reverse primer 5'-CCTCCCTCAAGTCTGTGAA-3'. Housekeeping gene GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as control, forward primer for GAPDH is 5'-TCCACCACCCTGTTGCTGTA-3', reverse primer for GAPDH is 5'-ACCACAGTCCATGCCATCAC-3'. PCR reactions were carried out in a Biometra Gradient Thermal Cycler for 30 cycles. Each cycle consisted of 94.0℃ for 30s, 56℃ for 30s and 72℃ for 30s. The reaction took place in a total volume of 25μL. PCR products were analyzed with 1.5% agarose gel electrophoresis and visualized by Gene finder staining under UV light. All primers were designed and synthesized by TaKaRa Company.

1.7 Statistical analysis

Data were analyzed using the paired *t* test and presented as the mean ± standard error. The significance level was set at 0.05. The SPSS13.0 software (SPSS, Inc., Chicago, Illinois, USA) was used for statistical analysis.

2 Results

2.1 XRD DLS and TEM examination

The XRD patterns (Fig. 1(a)) of sphere-like and rod-like particles indicated that no other phases but HA (01-074-0566) phase was found. As needle-like nano-HA were deposited on the titanium plate, the Ti phase also presented as shown in Fig. 1(a). It was also re-

vealed that rod-like and needle-like particles exhibited sharp diffraction peaks, indicating a high crystallinity, whereas the sphere-like particles had weak crystallinity. FT-IR results showed that there were no typical CTAB bands (2918, 2860 and 2944cm⁻¹) in Fig. 1(b). TEM results (Fig. 2) revealed that different morphologies of nano-HA were prepared successfully and dispersed stably in the medium system, and the characteristics of the sphere-like and rod-like particles (size, shape) were consistent with those described above respectively. The needle-like particles were (100 ± 20) nm in length and (20 ± 10) nm in width.

Figure 3 showed that the four kinds of particle size were uniform and no particle agglomerations were formed. The size distribution of the particles in the cell culture medium was consistent with TEM results.

2.2 Effect of nano-HA on U87 cells

In Fig. 4, it was showed that PEG-200 had no effect on U87 cells proliferation, however, different shapes of nano-HA all inhibited the proliferation of U87 cells. And their proliferation was inhibited by nano-HA in a dose-dependent manner as shown in Fig. 5. According to data analysis using SPSS 13.0, the IC50 values of nano-HA effect on U87 cells were 42.05μg/mL (needle-like),

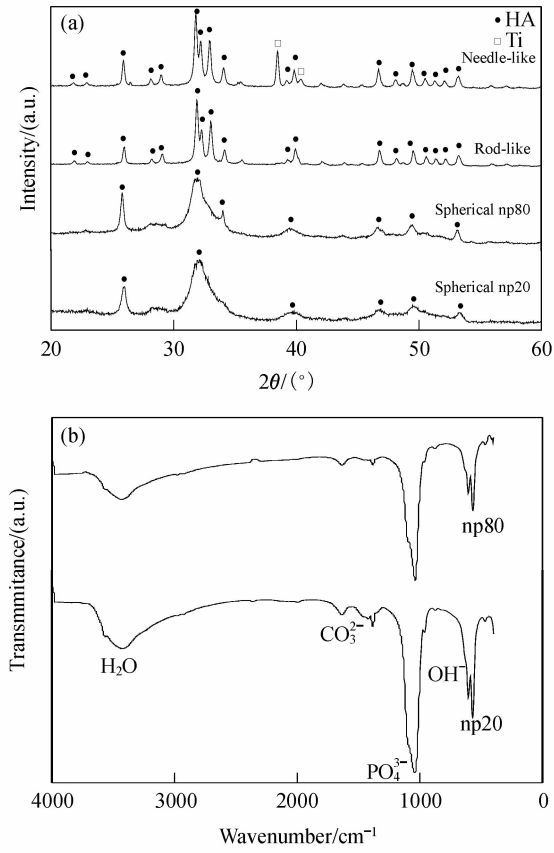


Fig. 1 (a) XRD patterns of nano-HA with different morphologies, (b) FTIR spectra of np20 and np80

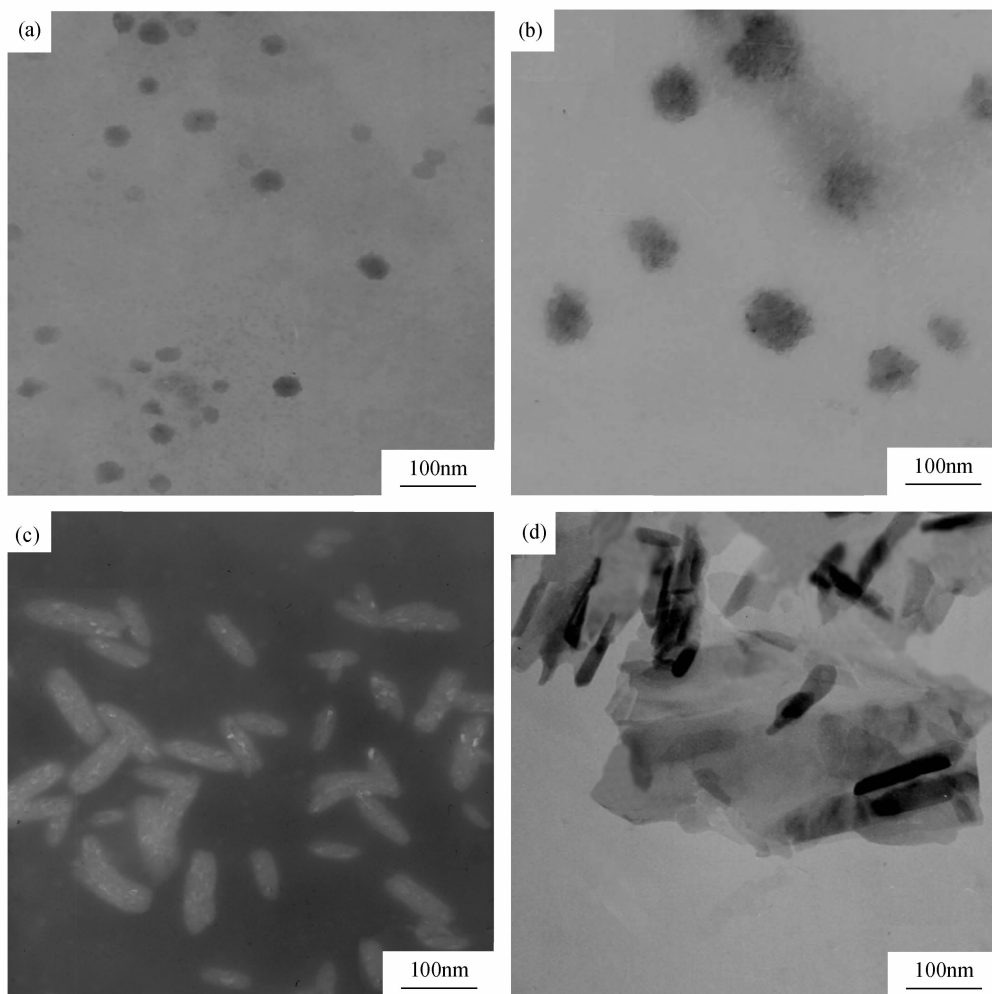


Fig. 2 TEM images of (a) sphere-like shape np20, (b) sphere-like shape np80, (c) rod-like, (d) needle-like in the medium system

54.58 $\mu\text{g/mL}$ (rod-like), 63.31 $\mu\text{g/mL}$ (np80), and 68.85 $\mu\text{g/mL}$ (np20), respectively. Needle-like nano-HA showed higher inhibitory rate than the others ($P < 0.05$). When treated with nano-HA at IC₅₀, the U87 cell viability was found less than 30% after 72h (Fig. 6), which was apparently lower than that at 48h. It was also noticed that needle-like nano-HA had higher IR than the others ($P < 0.05$). All above indicated that needle-like nano-HA had the most effective inhibition on U87 cells proliferation.

2.3 Toll-like receptor 4 expression in U87 cells

After exposure to 4 different shapes of nano-HA for 30min, the U87 cells were subjected to mRNA extraction and RT-PCR analysis. As shown in Fig. 7, Toll-like Receptor 4 expression was apparently high in U87 cells. After exposed to different shapes of nano-HA, TLR4 was down-regulated in all samples. Compared with the other shapes, needle-like nano-HA decreased the mRNA expression of TLR4 most significantly.

3 Discussion

The nano-HA ($\phi 20\text{--}120\text{nm}$) inhibit the proliferation of U87 cells, which may be induced by the chemical group and nano-structure of the HA crystal and the nano-dimension effect^[10,16].

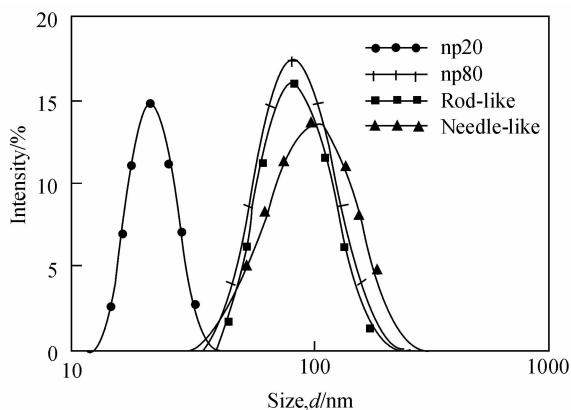


Fig. 3 Particle size distribution of nano-HA particles

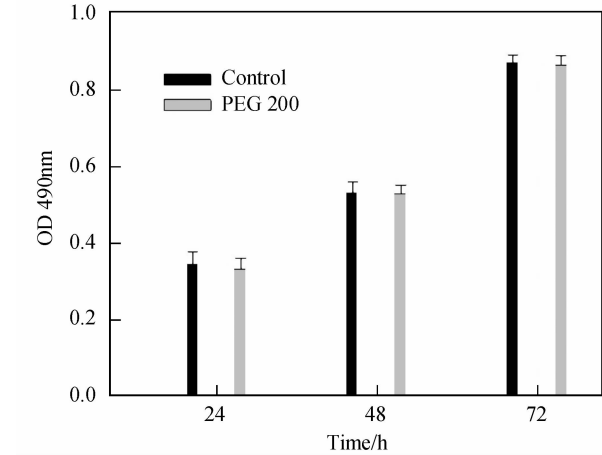


Fig. 4 Incubated with or without PEG-200(1.5mg/mL) U87 cells showed no statistical difference ($P > 0.05$) in cell viability (represented by OD490 values) at different time points between two groups. It meant that PEG-200 had no effect on U87 cells viability. bars, SD. t -test was used to compare between groups.

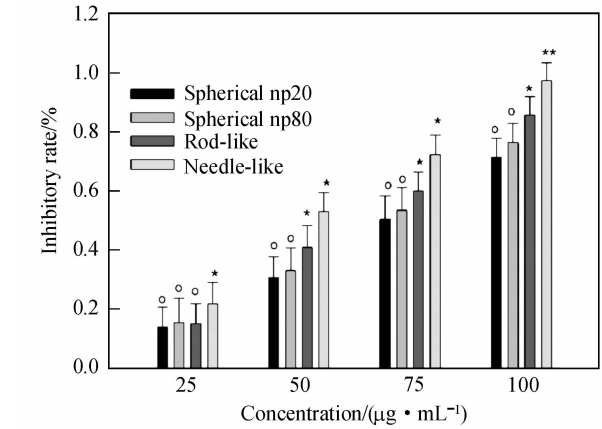


Fig. 5 The inhibitory rate ($IR, \%$) of U87 cells co-cultured with nano-HA evaluated with the MTT method The IR increases with the nano-HA concentration in a dose-dependent manner. t -test was used to compare between groups (statistical significance $^{\circ}P > 0.05$, $^*P < 0.05$. bars, SD.)

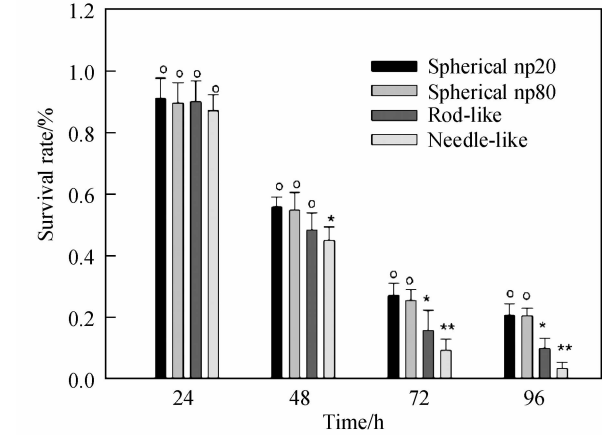


Fig. 6 The survival rate ($\%$) of U87 cells co-cultured with nano-HA The survival rate decreases with the time prolonged. t -test was used to compare between different groups (statistical significance $^{\circ}P > 0.05$, $^*P < 0.05$. bars, SD.)

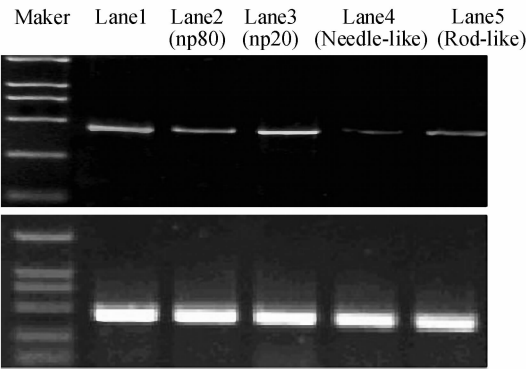


Fig. 7 Nano-HA down-regulate TLR4 expression

According to the MTT assay results (Figs. 5 and 6), it was proposed that the morphology of nano-HA was critical for its effect on cell proliferation. The mechanism that nano-HA inhibit cancer cells proliferation may be involved in the induction of inflammatory factor secretion. Laquerriere's group^[17-18] found that, compared with other shapes, needle-like HA induced the larger amount of production of TNF-alpha, IL-6, IL-10 and IL-18 by cells, and also induced the most significant up-regulated expression of MMPs, IL-1beta. Previous studies verified that HA particles only interact with TLR4⁺ monocytes/macrophages^[19]. Figure 7 indicated that TLR4 was also strongly expressed in U87 cells, which established the basement of their interactions. TLR4 were believed to be involved in tumor development and growth^[20-22] and promote the tumor cell proliferation. The TLR4 expression decreased significantly after adding the needle-like nano-HA, and this may be one mechanism for anti-proliferation effect of the needle-like particles.

4 Conclusions

Nano-HA of different shapes were prepared successfully and the nano-HA particles were stably dispersed in medium by using PEG-200 as dispersant. MTT assay revealed that three kinds of nano-HA could inhibit the proliferation of U87 cells, among which needle-like nano-HA showed highest anti-glioma ability. It was found that the morphology of nano-HA was critical for the anti-tumor ability. In addition, the alteration of gene expression may be another reason to explain the anti-tumor abilities of nano-particles. The expression of TLR4 decreased significantly when treated with needle-like nano-HA, which is potential for glioma treatment, however, the relationship between nano-HA and TLR4 still requires further studies.

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纳米羟基磷灰石对人脑胶质母细胞瘤 U87 细胞活性抑制效应的研究

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摘 要: 为了评价不同形貌的纳米羟基磷灰石粒子(nano-HA)对脑胶质母瘤细胞系 U87 细胞的作用,合成了三种不同形貌的 nano-HA,与 U87 细胞共培养,培养基中同时还添加了聚乙二醇(PEG),以保持 nano-HA 的稳定性.透射电镜(TEM)及动态光学散射(DLS)测定高度分散在培养基中 nano-HA 的稳定情况和粒子大小,MTT 法检测了 nano-HA 对 U87 细胞的作用.实验结果表明,nano-HA 可显著抑制 U87 细胞增殖;并且,针状纳米粒子的抑制能力要强于另外两种粒子.此外,通过 RT-PCR 检测发现不同形貌的 nano-HA 可不同程度的下调细胞的 Toll 样受体 4(TLR4)的表达水平,这可能是其具有不同生物学活性的原因之一.

关 键 词: 羟基磷灰石;胶质母细胞瘤;Toll 样受体 4;聚乙二醇

中图分类号: R739; TB321

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