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Preparation and *in vitro* activity of controlled release microspheres incorporating bFGF

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Objective: To study the preparative method of controlled release microspheres incorporating basic fibroblast growth factor (bFGF) and the bioactivities of bFGF, which were released from bFGF microspheres, on the cultured Schwann cells.

Methods: bFGF was microcapsulated with the multiple emulsion encapsulative method using polylactic-co-glycolic acid (PLGA) as coating material. Its morphology, particle size distribution, drug loading, enveloping rate and *in vitro* release property were studied. The cultured Schwann cells were grouped according to the different ingredients being added to the culture medium of bFGF group or bFGF-PLGA group. Then the cytometry, cytoactivity detection and mitotic cycle analysis of Schwann cells were performed.

Results: The morphology and the particle size distribution of the bFGF-PLGA microspheres were even and good. The drug loading and enveloping rate of microspheres were $(27.18 \times 10^{-3})\% \pm (0.51 \times 10^{-3})\%$ and $66.43\% \pm 1.24\%$. The release property of microspheres *in vitro* was good and the overall release rate was 72.47% in 11 days. The *in vitro* cellular study showed that: at the first 2 days of plate culture, the cell number and viability of the bFGF group were statistically higher than the bFGF-PLGA group; at the 3rd and 4th days of plate culture, the cell number and viability of bFGF and bFGF-PLGA groups showed no difference; at the 6th and 8th days of the plate culture, the cell number and viability of the bFGF-PLGA group were statistically higher than the bFGF group. By flow cytometry examination, at the 2nd day of plate culture, the G2/M + S percentage of bFGF group was statistically higher than the bFGF-PLGA group, at the 4th and 8th days of plate culture, the G2/M + S percentage of the bFGF-PLGA group was statistically higher than the bFGF group.

Conclusions: It is practical to prepare the bFGF-PLGA microspheres with the multiple emulsion encapsulative method. bFGF-PLGA microspheres can preserve the bioactivities of bFGF effectively and promote the proliferation of Schwann cells in a long period because of the controlled release of bFGF from the microspheres.

Key words: Fibroblast growth factors; Microspheres; Schwann cells

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Basic fibroblast growth factor (bFGF) has been proved to have mitogenic and differentiation-promoting effect on neuroectodermal cells, and it has great applicable potentials in the research field of nerve regeneration.[1,2] However, because of the instability in the heat and acid environment, along with a short half-life time ($T_{1/2}$) of 3-5 minutes *in vivo*, bFGF cannot satisfy the need for clinical applications either universally or locally. So it is meaningful to find out a controlled release agent of bFGF by which to maintain a prolonged effective drug concentration and improve nerve regeneration.[3,4]

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In this study, polylactic-co-glycolic acid (PLGA) was used as the coating material, and a multi-emulsion encapsulating mechanism was used in the preparation of controlled release microspheres incorporating bFGF. Its morphology, particle size distribution, drug loading, enveloping rate and *in vitro* release property were studied. In addition, cytometry, cytoactivity detection and mitotic cycle analysis were performed in order to achieve a preliminary idea about the bioactivity of the bFGF being released from the controlled release microspheres through adding the bFGF microspheres into the culture medium of the Schwann cells.

METHODS

Reagent and equipment

Freeze-dried bFGF powder (EssexBio, China), Polylactic-co-glycolic acid copolymer (Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences, Mv 12 000, 50:50), Rabbit polyclonal

antibody against bovine bFGF (Sigma, USA), Goat antibody against rabbit IgG marked with horseradish peroxidase enzyme (Beijing Zhongshan Biotech Ltd Co., China), Polyclonal antibody against S-100 protein (Beijing Zhongshan Biotech Ltd Co., China), Dulbecco's Modified Eagle's Medium (DMEM, Gibco, USA), Type-Ⅱ Collagenase (Sigma, USA), Trypsin (Sigma, USA), Collagen (Sigma, USA), Methylthiazolyl tetrazolium (MTT Sigma, USA).

L8-80M speeding refrigeration centrifuge (USA), AMRAY electronic scanning microscopy (USA), Malvern mastersizer 2000 laser particle size distribution tester (USA), Lyophilizer (Savant Modulyo, USA), Bio-RAD Model 550 photodensitometer (USA), Flow cytometer (Elite SP, USA), Class Ⅱ A/B3 biological safety cabinet (USA), CO_2 gas incubator (Sanyo, Japan), Refrigeration centrifuge (Megafuge 1.0R, USA).

Preparation of bFGF-PLGA controlled release microspheres

According to the optimized prescription obtained from the preliminary test, PLGA was dissolved by the dichloromethane solution. Once completely dissolved, the bFGF solution was added and the mixture was homogenized by ultrasonic wave into emulsion. 6% polyvinyl alcohol (PVOH) solution was used as the aqueous phase dispersed medium, whose inorganic salt concentration was 2%. 2 ml PVOH solution was then mixed into the emulsion. After completely stirred, the emulsion was added into 8 ml polyvinyl alcohol (PVOH) solution of the same concentration, stirred by magnetic force under ambient temperature until the complete volatilization of the dichloromethane, and the colloid solution of bFGF-PLGA controlled release microspheres was then obtained. Proper amount of 1% lactose as frame materials was added into the microsphere solution which was conserved under freeze-dried circumstance.

Morphology and particle size distribution of bFGF-PLGA microspheres

Electronic scanning microscopy was used to observe the morphology of bFGF-PLGA microspheres. Malvern laser particle size distribution tester was used to determine the particle size of the bFGF-PLGA microspheres. The size distribution of the bFGF-PLGA microspheres was calculated according to the Chinese Dispensary 2000: size distribution = (D90-D10)/D50.

Examination of drug loading and enveloping rate of the bFGF-PLGA microspheres

The enzyme linked immunosorbent assay (ELISA) was used to examine the absorbency (*A* value) of the standard bFGF solution. The *A* value was used for the logarithm linear regression of the standard bFGF solution and the equation was established. Certain amount of newly prepared bFGF-PLGA microspheres was obtained and centrifuged at 40 000 r/min under 4℃ for 2 hours. The ELISA was used to determine the *A* value of the supernatant fluid and the floating drug load (the drug load in the liquid medium) was calculated according to the regression equation. The drug loading and enveloping rate were then obtained according to the formulations.

***In vitro* drug release of bFGF-PLGA microspheres**

According to the drug loading, bFGF-PLGA microspheres which contain 1.5 μg bFGF were added into centrifugal tubes ($n = 3$). Then 2 ml normal saline was added into each tube and oscillated with constant temperature and speed (37℃ and 20 r/min). The concentrations of bFGF in the supernatant fluid from the centrifuge tubes were measured at 12, 24, 36, 48, 60, 72, 84 hours and 5, 6, 7, 8, 9, 10, 11 days respectively. The original volumes were restored after sampling. The bFGF-PLGA microspheres releasing curve was drawn according to the accumulated release rate and time data.

Cultivation, identification and grouping of Schwann cells

Primary Schwann cells, which were obtained from bilateral sciatic nerves of newborn New Zealand rabbits, were cultivated using a combination of enzymic digestion and repeated attachment method. The cultivated Schwann cells were identified by immunohistochemistry staining using anti-S-100-protein polyclonal antibody and then subcultured for the subsequent use. Experiment grouping: Group A contained 10% calf serum and DMEM with 50 ng/ml bFGF; Group B contained 10% calf serum and DMEM with bFGF-PLGA microspheres which contain 50 ng/ml bFGF.