

Research Article

Preparation, Characterization, and Bioactivity of Chitosan Microspheres Containing Basic Fibroblast Growth Factor

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The aim of this study is to evaluate, prepare, and characterize bioactivity of chitosan microspheres loaded with bFGF for providing sustained release of bFGF. Porous chitosan microspheres were prepared by freeze-drying process based on the interaction between chitosan and tripolyphosphate (TPP). The bFGF-loaded chitosan microspheres were well interconnected and have a narrow size distribution, spherical shape, and positive surface charges. The bFGF-loading capacity and encapsulation efficiency were 7.57 mg/g and 95.1%, respectively. Results of *in vitro* release showed that the extent of release was 82.1% at Day 25. Schwann cells were used as an *in vitro* model for cell response to bFGF and bFGF-loaded chitosan microspheres. Results indicated that the number, cell viability, and percentage of cells G2/M+S phase in the bFGF groups are higher than those in the bFGF-loaded chitosan microspheres groups before culturing for 2 days. However, the number, cell viability, and percent of cells G2/M+S phase in the bFGF-loaded chitosan microspheres groups are significantly higher than those in the bFGF groups after culture for 4 and 8 days. These findings indicated that bFGF-loaded chitosan microspheres may help to decrease the release of bFGF and provide a suitable three-dimensional environment for cell growth and proliferation.

1. Introduction

Fibroblast growth factors (FGFs) constitute a family of heparin binding polypeptides, which are involved in the regulation of biological responses, such as growth, differentiation, and angiogenesis [1]. Basic fibroblast growth factor (bFGF) is a member of the FGF family that comprises nine members. This FGF prototype has pleiotropic effects in different cell and organ systems. bFGF is a potent angiogenic molecule that *in vivo* and *in vitro* stimulates smooth muscle cell growth, wound healing, and tissue repair [2, 3]. In addition, bFGF may stimulate hematopoiesis and may play an important role in the differentiation and/or function of nervous system, eye, and skeleton [4]. However, the bioactivity of bFGF cannot always be expected when it is injected into the body by soluble form, because of its short duration of retention at wound sites and short half-life caused by susceptibility to enzymatic and thermal degradation *in vivo* [5]. Poor stability is considered to be one of the major factors that limit pharmacological applications of proteins, generating problems in formulation,

administration, and storage. So, an effective delivery system of bFGF is required to overcome the shortcoming of bFGF application in a clinical setting.

Recently, a number of strategies and delivery methods have been designed and evaluated for the reportedly poor thermal stability and a relatively short *in vivo* half-life [6, 7]. One of the approaches for enhanced thermostabilization of proteins is to alter the microenvironment using additives. Additives may modify the solvent structure such as sugar, arginine, heparin, and so forth, which have been tried with considerable success for understanding the mechanism of thermal inactivation in some growth factors [5]. Another option to enhance *in vitro* and *in vivo* efficacy of growth factors is to incorporate them into polymeric biomaterials, like scaffold, microsphere, gel, and so forth, which may maintain their stability and control their release kinetics. The use of microsphere-based therapy allows drug release to be carefully tailored to the specific treatment site through the choice and formulation of various drug-polymer combinations. The total dose of medication and the kinetics of release are the

variables, which can be manipulated to achieve the desired result. Using innovative microencapsulation technologies, and by varying the copolymer ratio, molecular weight of the polymer, and so forth, microspheres can be developed into an optimal drug delivery system which will provide the desired release profile. Microsphere-based systems may increase the life span of active constituents and control the release of bioactive agents [8–10].

Chitosan is a biodegradable natural polymer with potential for pharmaceutical applications for its biocompatibility, high charge density, nontoxicity, and mucoadhesion. It not only improves the dissolution of poorly soluble drugs but also exerts a significant effect on fat metabolism in the body [11, 12]. Thus, chitosan possesses suitable properties as a carrier for microsphere drug delivery. Chitosan microspheres are used to provide controlled release of many drugs and to improve the bioavailability of degradable substances such as protein or enhance the uptake of hydrophilic substances across the epithelial layers. Chitosan has also been used as a potential carrier for prolonged delivery of drugs, macromolecules, and targeted drug delivery. Magnetic chitosan microspheres used in targeted drug delivery are expected to be retained at the target site capillaries under the influence of an external magnetic field [11]. Various devices, including microspheres, scaffolds, and hydrogels, have been used to bind and release angiogenic growth factors, such as vascular endothelial growth factor (VEGF), acidic and basic fibroblast growth factor (aFGF and bFGF), and platelet-derived growth factor (PDGF) [6, 7]. These reports will help to understand the potential application of microspheres and deliver growth factors in tissue repair engineering. However, further extensive research will be carried out to exploit chitosan as a growth factor carrier to attain the desirable results. Thus, bFGF was chosen to incorporate into the chitosan microspheres based on the emulsion cross-linking method. The morphology, particle size and distribution, bFGF-loading capacity, encapsulation efficiency, and *in vitro* release rates were analyzed using electron microscopy, laser particle size analyzer, potentiometric analyzer, and ELISA methods. In addition, the bioactivity of released bFGF was evaluated by measuring ability of the released bFGF to stimulate proliferation of Schwann cell.

2. Materials and Methods

2.1. Chemicals and Materials. Chitosan, sodium tripolyphosphate, liquid paraffin, sorbitan monopalmitate 80, and so forth were obtained from Chengdu KeLong Chemicals (Chengdu, China). Human bFGF was supplied by PrimeGene (Shanghai PrimeGene Bio-Tech Co., Ltd., Shanghai, China). Antibodies used in the present work were purchased from Beijing Zhongshan Golden Bridge Biotechnology Co., Beijing, China. Dulbecco's modified essential medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco Invitrogen Corporation (Carlsbad, CA, USA). All other solvents used in this study are of analytical grade.

2.2. Preparation of bFGF-Loaded Chitosan Microspheres. Chitosan solutions with concentrations of 1.2 and 1.5% (w/v)

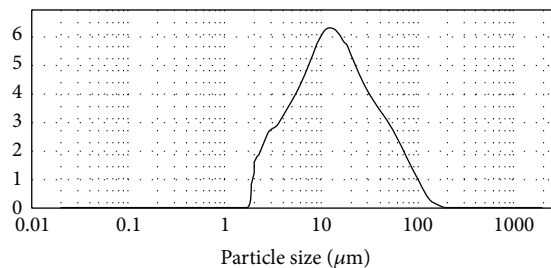


FIGURE 1: Particle size of bFGF-loaded chitosan microspheres.

were prepared by dissolution of chitosan flakes in 0.35 M acetic acid and were filtered through 0.45 μm membrane filter (Millipore) for removing any nondissolved residue particles. bFGF-loaded chitosan microspheres were prepared *via* freeze-drying method which was described as follows. Firstly, the concentrations of chitosan (1.2% and 1.5%) and sodium tripolyphosphate (0.6% and 1%) were selected based on the particle size and distribution of microspheres, and the experiments were designed as four groups. Reports from particle size analysis showed the concentrations of chitosan and sodium tripolyphosphate use 1.5% and 0.6% as optimized formulation for the preparation of microspheres. The particle size is about 21.554 μm with a single peak, and the sizes in the other three groups show bimodal shape (Figure 1). Second, the bFGF-loaded chitosan microspheres were prepared based on the optimized formulation. The bFGF solution (1 mg/mL, 0.15 mL) was mixed with chitosan solution (1.5%, 1 mL), and removed air bubbles in the solution using ultrasonic in the bath. The sodium tripolyphosphate (0.6%, 0.4 mL) was added to the above mixture, and then the mixtures were stirred at 500 rpm for 60 min. The chitosan-bFGF solution was added dropwise to liquid paraffin (6.18 mL, containing 0.18 mL SP-80) stirring at 500 rpm for 60 min and emulsified stirring at 500 rpm for 48 h at 37°C. The samples were centrifuged at 2,000 rpm for 5 min at 4°C, and the precipitate was washed several times with petroleum ether. The precipitates were then freeze-dried for 24 h at room temperature, and the powder was collected and stored at 4°C for further analysis.

2.3. Analysis of Particle Size and Morphology. The particle size and distribution of bFGF-loaded chitosan microspheres powder were analyzed using laser particle size and zeta potential analyzer. The morphology of microspheres powder was observed by scanning electron microscopy after spraying.

2.4. Analysis of bFGF-Loading and Entrapment Efficiency of Microsphere. The bFGF-loading capacity and encapsulation efficiency were carried out according to the instructions of ELISA kit. The standard equation was calculated based on the absorbance values of microspheres at 450 nm. The bFGF-loaded chitosan microspheres powder (15 mg) was placed in the container containing PBS buffer solution (pH 7.4, 50 mL) and filtrated using filter membrane (0.45 μm). The bFGF-loading capacity and encapsulation efficiency were calculated

based on the absorbance values in the washing liquid. The bFGF-loading capacity and encapsulation efficiency were calculated for each treatment using the following equations: bFGF-loading capacity = (drug loading dosage – drug amount in the wash solution)/total amount in the microspheres; the encapsulation efficiency (%) = (drug loading dosage – drug amount in the wash solution)*100%/total amount in the microspheres. The experiments were arranged in a completely randomized design with three replicates per treatment.

2.5. In Vitro bFGF Release Studies. Sixty milligrams of bFGF-loaded chitosan microspheres was placed in the dialysis bag and suspended in 60 mL of PBS buffer (pH 7.4) as a medium at 37°C with mild agitation (100 rpm). At several intervals, the supernatants were collected by centrifugation, and the amount of the released bFGF was determined by using ELISA method. The cumulative release rate was calculated for each treatment using the following equation: cumulative release rate (%) = $W1/W2 \times 100\%$, where $W1$ is bFGF amount in the wash solution and $W2$ is the bFGF amount in the microspheres. The burst release rate is expressed as the cumulative release rate in the first day. All release studies were repeated three times and the results were reported as average values.

2.6. Effects of bFGF-Loaded Chitosan Microspheres on the Cell Number, Cell Viability, and Cell Phase. The experiments were separated into two groups. One group was allowed to culture with Dulbecco's modified essential medium (DMEM) containing 10% fetal bovine serum (FBS) plus 50 ng/mL bFGF as control. Another group was cultured with DMEM containing 10% FBS plus 50 ng/mL bFGF of microspheres. Widely used Schwann cells were chosen as *in vitro* experiment system. The Schwann cells were plated in 24-well plates at 1.0×10^4 cells/mL. The cells in two groups were collected at Days 1, 2, 3, 4, 6, and 8, respectively. The cell number was counted using cell counting plate. The cell viability was followed by MTT assay. In brief, the tetrazolium salt (2 mg/mL, 50 μ L) was added to the wells before collection, and the cells were cultured for 4 h. Then, the dimethyl sulfoxide (150 μ L) was added in the wells and stirred for 10 min. The absorbance values in the wells were measured at 490 nm using a microplate reader (Bio-Tek. Instruments, Winooski, Vermont, USA). After culture, the cells were collected at Days 2, 4, and 8, and the cell phases were measured using flow cytometry. Then, the cell percentages of G2/M+S phase were calculated based on the analysis results. All release studies were repeated three times and the results were reported as average values.

2.7. Statistical Analysis. All treatments were arranged in a completely randomized design with three replicates. Data were expressed as mean \pm SD. Statistical significance was evaluated with Student's *t*-test, and differences were considered significant if *P* values were less than 0.05.

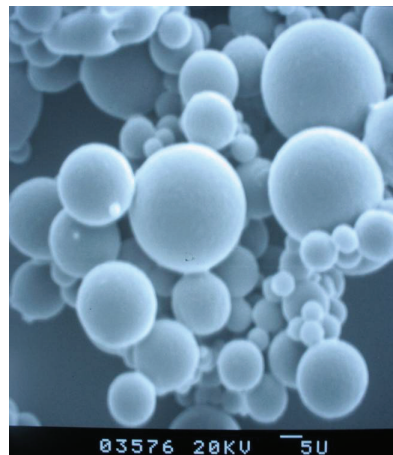


FIGURE 2: Structural properties of bFGF-loaded chitosan microspheres measured by scanning electron microscopy (SEM, $\times 4000$).

3. Results

3.1. Morphology of bFGF-Loaded Chitosan Microspheres. As shown in Figure 1, the average diameter of 35.335 μ m of bFGF-loaded chitosan microspheres was observed using laser particle size and potential analyzer. Its size showed relative uniform distribution. As shown in Figure 2, the bFGF-loaded chitosan microspheres shown have interconnected structure and morphology, which have round smooth surface, no obvious pores, and good dispersity based on the results of scanning electron microscopy.

3.2. The bFGF-Loading Capacity, Encapsulation Efficiency, and In Vitro bFGF Release Studies of bFGF-Loaded Chitosan Microspheres. After measure and calculation, the concentration of bFGF in the washing solution was approximately 2.43 μ g/mL, and the total amount of bFGF was approximately 7.29 μ g. Based on the calculated equations, the bFGF-loading capacity and encapsulation efficiency were 7.57 mg/g and 95.1%, respectively. bFGF release from the bFGF-loaded chitosan microspheres started with an initial burst effect and continued with a controlled and steady release period. Total release of bFGF from the bFGF-loaded chitosan microspheres lasted as long as 24 days. At Day 1, bFGF was released by 180.8 ng/mL, and the burst release rate in the first day was 18.1%. The concentrations of bFGF reached 820.5 ng/mL from Day 2 to Day 24, and the average increase concentration was 53.3 ± 12.8 ng/mL every two days. The cumulative release rate was 82.1% from Day 2 to Day 24.

3.3. Effects of bFGF and bFGF-Loaded Chitosan Microspheres on the Cell Number. As shown in Figure 4, the cell number in the microspheres groups at different cultured times shows significant difference compared to that of the control groups. The cell number in the microspheres groups is less by about 35.5% and 36.4% at Days 1 and 2 compared with the control groups. At Days 3 and 4, the number in the microspheres groups showed no significant difference compared with the control groups, respectively. The values in the microspheres

groups are significantly higher than the control by 25.8% and 22.4% at Days 6 and 8, respectively.

3.4. Effects of bFGF and bFGF-Loaded Chitosan Microspheres on the Cell Viability. The effects of bFGF and bFGF-loaded chitosan microspheres on the cell viability are shown in Figure 5. The cell viability in the microspheres groups decreased by 26.8% and 36.2%, respectively, compared with the control after 1 and 2 days of culture. After 3 and 4 days, the microspheres groups experienced no difference compared with the control groups, respectively. The cell viability of the microspheres groups was 33.1% and 22.3% higher than the control groups after 5 and 6 days, respectively.

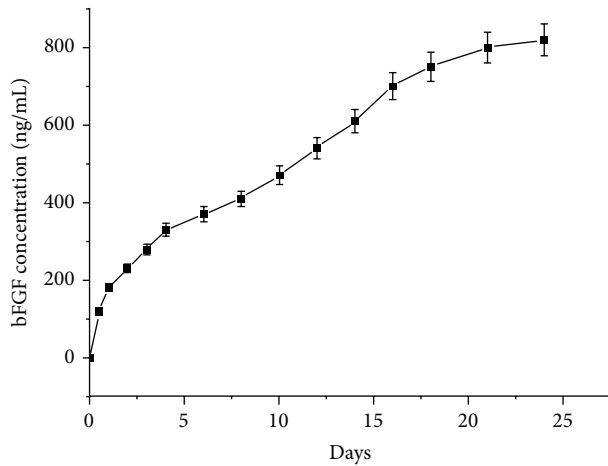
3.5. Effects of bFGF and bFGF-Loaded Chitosan Microspheres on the Cell Phases. The effects of bFGF and bFGF-loaded chitosan microspheres on the percentages of G2/M+S phase were shown in Figure 6. As shown in Figure 6, the cell percentages of G2/M+S phase showed significant different after culturing for 2, 4 and 8 days between microspheres and control group. At Day 2, the percentages in the microspheres groups were significantly less by 42.1% compared to the control groups. However, the percentages in the microspheres groups were significantly higher by 51.8% and 97.4% compared with the control groups at Days 4 and 8, respectively.

4. Discussion

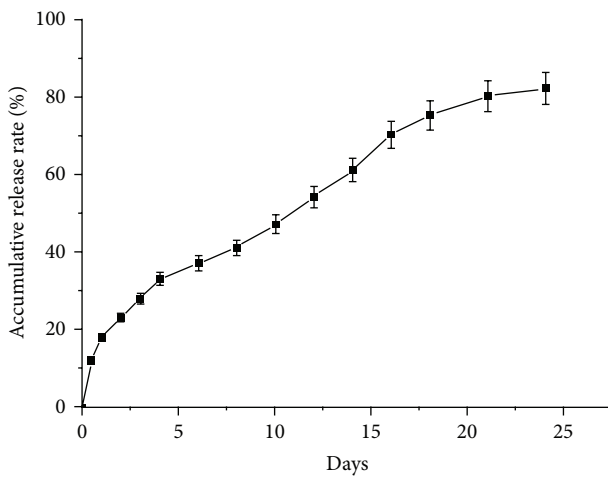
A multifunctional role of bFGF is suggested by many different receptor phenotypes expressed in various cell types. bFGF stimulates the growth of fibroblasts, myoblasts, osteoblasts, neuronal cells, endothelial cells, keratinocytes, chondrocytes, and many other cell types. It is assumed that bFGF will be of potential interest as a factor promoting wound healing and revascularization of tissues. Animal experiments with bFGF have shown that it promotes endosteal, but not periosteal, bone formation and bFGF may thus be a potential agent for treating osteoporosis, which may increase bone mass without causing outward deformation of the skeletal bones [1–3]. However, bFGF is a very unstable protein. At alkaline pH, covalent aggregation of bFGF for thiol-disulfide exchange or β -elimination was observed. Under acidic conditions, degradation products of bFGF caused by peptide bond hydrolysis deamidation were generated. In addition, bFGF has a very short half-life *in vivo* at physiological pH and temperature [5], and this limits its therapeutic value for frequent administration. Thus, the sustained-release formulations will help to maintain the bFGF activity and/or improve the stability of bFGF and to prolong its effects *in vivo*. Chitosan is a partially deacetylated polymer obtained from the alkaline deacetylation of chitin, which is a glucose-based, unbranched polysaccharide. Chitosan exhibits a variety of physicochemical and biological properties resulting in numerous applications in fields. In addition to its lack of toxicity and allergenicity, its biocompatibility, biodegradability, and bioactivity make it a very attractive substance for diverse applications as a biomaterial in the pharmaceutical and medical fields [13, 14]. Chitosan microspheres are used

to provide controlled release of many drugs, such as insulin, antibiotics, growth factors, and so forth, which improve the bioavailability of degradable substances such as protein or enhance the uptake of hydrophilic substances across the epithelial layers [11]. The preparation methods of chitosan microspheres mainly include phase separation, ion-induced emulsion crosslinking, spray drying, and so forth [12]. In the present study, the emulsion crosslinking method was selected to prepare bFGF-loaded chitosan microspheres for the unstability of bFGF to heat and organic reagents. The preparation of microspheres may be affected by a variety of factors, like chitosan concentration, crosslinking agent, emulsion temperature, stirring speed, and so forth. Shu and Zhu reports indicated that microspheres have a good appearance of the sphere using crosslinker of sulfate salt, citrate salt, and TPP, and the microspheres have better mechanical properties, swelling resistance, and drug release properties using TPP as crosslinker [15]. TPP have some advantages, such as mild reaction conditions, simply operation, and little effects on bFGF activity. Thus, the present study selected TPP as a crosslinking agent for the preparation of microspheres. The present results showed that bFGF-loaded chitosan microspheres have a good appearance of the sphere, smooth surface, and good dispersion using 1.5% of chitosan concentration and 0.6% of TPP (Figure 1). In addition, our results also showed that microspheres are easily dispersed and unevenly distributed with the increasing concentrations of chitosan and viscosity of chitosan solution (data not shown). The particle sizes are relative to stirring speed except for other parameters. The particle size gradually decreased with the increasing stirring speed, and the higher constant stirring helped to form a uniform particle size distribution of microspheres. Thus, the 100 rpm of stirring speed was selected during the preparation process of microspheres. The microspheres are easily stuck together and are difficult to disperse for the water-in-oil system of chitosan microspheres. Thus, the higher temperatures help the evaporation of water, and the experiment temperature was selected at 37°C for reaction temperature. The Span-80 was selected as emulsifier in this study, which may form a protective layer on the surface of microspheres to prevent the adhesive interaction of microspheres. The results of Zeng et al. [16] showed that the particle size decrease and even distribution are observed with the increasing emulsifier concentration. However, the excessive emulsifier will be difficult for elution and will result in producing certain toxicity. Thus, the 3% Span-80 was selected as the present experiment condition.

Drug loading capacity and encapsulation efficiency are important indicators of quality to drug-loaded microspheres, which were affected series of factors. The most important factor is the preparation process of microspheres [11, 12]. The present study suggested that the bFGF-loading capacity and encapsulation efficiency reach 7.57 mg/g and 95.1% (Figure 3), respectively, which showed that the preparation process of bFGF-loaded chitosan microspheres is effective and feasible using emulsifying crosslinked method. Burst-release is the rapid release of drug from the formulation occurring in the initial 24 h of elution. There are generally burst release phenomena to biodegradable microspheres for protein and/or



(a)



(b)

FIGURE 3: Changes of bFGF release rates from bFGF-loaded chitosan microspheres. Results are averages of three independent experiments. Data represent mean \pm SEM.

peptide delivery. This event causes undesirable loss and potentially toxic levels of therapeutic from the microsphere. Thus, modulation of release is of particular importance in these studies because little is known regarding the therapeutic dose of protein and/or peptide in humans [12, 17]. Burst release rate is an important indicator to assess microsphere drug delivery system, and the values are approximately 20~30%. In the present study, *in vitro* release process of the bFGF-loaded chitosan microspheres showed relatively stable and smooth trend, and the cumulative burst release rate is 18.1% in the first day. The cumulative release rate is 82.1% from Day 2 to Day 24, and the even value is approximately 53.30 ng/mL every two days. These results indicated *in vitro* release process of bFGF-loaded chitosan microspheres is slow; that is, the increased concentration of bFGF is lower every two days.

Schwann cells (SCs) are generated from neural crest cells and undergo extensive migration, proliferation, and maturation before they terminally differentiate. SCs are valid candidates for cell therapy of PNS injuries, being the glial as

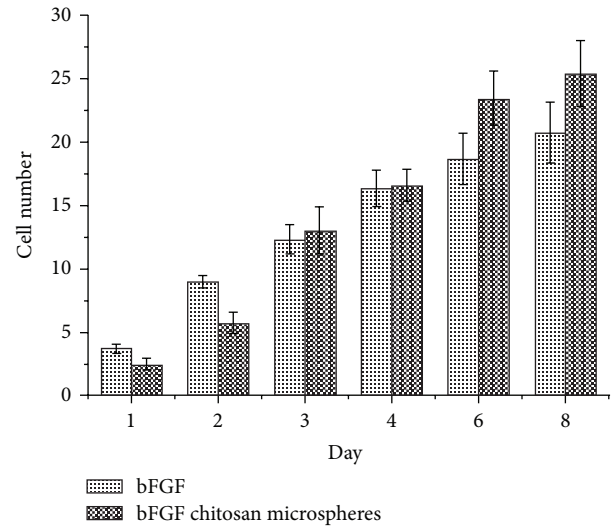


FIGURE 4: Effects of bFGF and bFGF-loaded chitosan microspheres on the cell number. Results are averages of three independent experiments. Data represent mean \pm SEM.

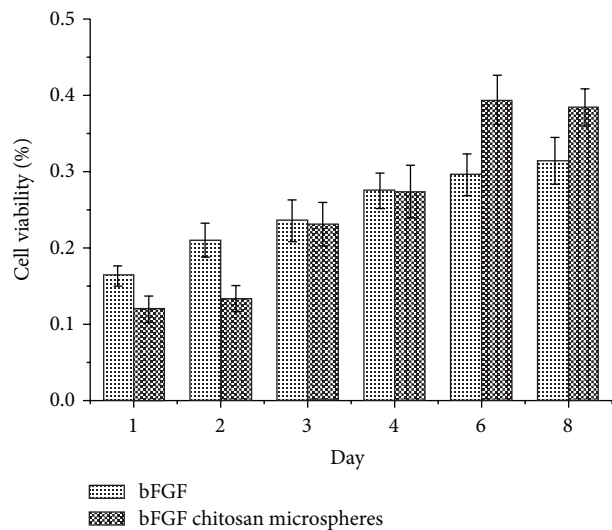


FIGURE 5: Effects of bFGF and bFGF-loaded chitosan microspheres on the cell viability. Results are averages of three independent experiments. Data represent mean \pm SEM.

well as myelinating cells in the PNS and playing a crucial role both in supporting peripheral axon regeneration and remyelination after injury [18, 19]. It has been well established that artificial nerve grafts filled with naive SCs from neonatal stimulate peripheral nerve fiber regeneration. The growth-promoting effect is thought to occur as a result of the SCs ability to secrete a range of trophic factors including bFGF [20]. Wang et al. reported that the expression and secretion of sustained high levels of bFGF were observed in *in vitro* culture after exogenous bFGF mRNA modified Schwann cells. In addition, bFGF have mitogenic activity on the cultured Schwann cell. After bFGF binding to receptor of target cells, the transcription levels of ribosome gene will

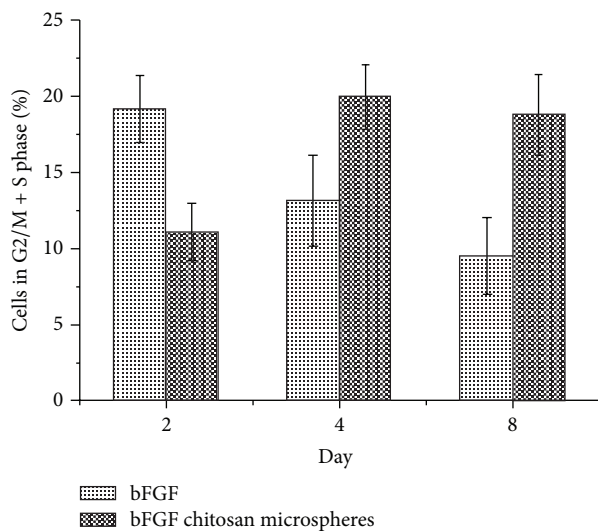


FIGURE 6: Effects of bFGF and bFGF-loaded chitosan microspheres on the cell phases. Results are averages of four independent experiments. Data represent mean \pm SEM.

accelerate by the influence of RNA polymerase I, and the cells were promoted G_1 -to-S phase and G_1 -to-S phase transition, which will shorten the G_1 phase and promote cell division and proliferation [21]. In the present study, results from cell number, cell viability, and cell phase suggested that the mitogenic activity in the bFGF-loaded chitosan microspheres groups is less than that in the bFGF groups at Days 1 and 2, but the mitogenic activity in the bFGF-loaded chitosan microspheres groups is significantly higher than that in the bFGF groups at Days 6 and 8 (Figures 3, 4, and 5). In the bFGF and bFGF-loaded chitosan microspheres, the total amount of bFGF is the same. At Days 1 and 2, the bFGF concentration in the bFGF group is significantly higher than that in the bFGF-loaded chitosan microspheres for sustained release process of microspheres groups. This may be consistent with the results of mitogenic activity. However, the bFGF concentration in the microspheres group gradually increased with the sustained release and increasing time, and the mitogenic activity in the microspheres group is significantly higher than that in the control groups at Days 6 and 8. These findings demonstrated that bFGF-loaded chitosan microspheres provide a suitable environment supporting the cellular structure, proliferation, and mineralization. However, the differentiation mechanism of Schwann cells by which the bFGF-loaded chitosan microspheres environment promoted cell proliferation is complex and needs further study.

5. Conclusion

In summary, a simple operation and emulsion crosslinking method for the preparation of bFGF-loaded chitosan microspheres has been developed, and the microspheres had smooth sphere surface, uniform distribution, and dispersion. Results of *in vitro* release showed that release rate is relatively

stable and maintains the biological activity of bFGF. In addition, bFGF-loaded chitosan microspheres may be developed for the deployment of a controlled release growth factors delivery system to promote growth and mitogenic activity of cells. These findings suggest that bFGF-loaded chitosan microspheres are a promising biomaterial for improving skin regeneration efficacy and promoting vascularization.

Conflict of Interests

There is no conflict of interests to declare, and each author certifies that they have no commercial associations that might pose a conflict of interests in connection with this paper.

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