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Research Article

The Preparation and *In Vitro* Evaluations of a Nanoscaled Injectable Bone Repair Material

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There are usually two forms of bone repair materials, block and granular, for common clinical use. This paper describes a novel injectable material, nano-HA/collagen/alginate (nHAC/Alg) composite biomaterial, including its preparation and evaluations *in vitro*. Based on the idea of bionics and the study of collagen/calcium phosphate salt composite materials, the injectable bone repair material was developed. Then, human bone marrow stem cells (hBMSCs) were cultured on the nHAC/Alg material. The cell attachment, proliferation, and differentiation were evaluated with inverted microscope, scanning electron microscope, laser scanning confocal microscope, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) analysis, and alkaline phosphatase (ALP) test. The results showed that nHAC/Alg not only had no negative effect on cellular functions but also promotes cell proliferation and differentiation into osteogenic cells, which suggests that the nanoscaled injectable bone repair material has good clinical application prospects for bone repair.

1. Introduction

Bone substitute is a common tool used in orthopaedics and dentistry to bridge bone deficiencies that may be due to trauma, disease, or surgery [1]. Nowadays, the design and the fabrication of injectable systems are becoming more and more important in minimally invasive surgical procedures and are given a special attention [2–8]. In particular the injectable bone substitutes (IBSs) that contained cements, pastes, putties, and gels have gained extreme interests in the field of bone regeneration. Compared to solid and preset scaffolds, IBSs are using a syringe through a very small incision, which could reduce patient malaise and shorten the time of surgical and recovery procedure. Meanwhile, IBSs could be a good filler of irregularly shaped bone defects and an intimate contact between the injected material and the bone surface [9]. In 2006, Liu and his team developed a novel injectable bone substitute material which consists of chitosan, citric acid, and glucose solution as the liquid phase and tricalcium phosphate powder as the solid phase [10]. In 2009, a paper presented an improved sol-gel technique, which includes a premineralization step of atelocollagen providing a suspension of microfibrils in which the synthesis of the inorganic component was conducted at low temperature for the preparation of hydroxyapatite-atelocollagen composites suitable as precursor, for osseous regeneration [11]. In 2011, Ghanaati and coworkers described an injectable bone substitute being composed of beta-tricalcium phosphate granules, methylcellulose, and hyaluronic acid [12]. In 2012, Hu and his team composited a kind of compound of calcium sulfate hemihydrate (CSH) and nano-HA/collagen (nHAC) as an injectable and self-setting scaffold in bone graft substitute [13]. In 2013, D'Este and his members showed a well-established trend for bone substitutes, called combination of hydrogels and calcium phosphate particles [14], while Lian and his members developed a kind of mineralized collagen based composite nano-hydroxyapatite/collagen/poly called VCM/nHAC/PLA which showed good biocompatibilities

in vitro and *in vivo* [15]. In 2014, Chen and his team developed the nHAC/CSH composite as an injectable bone repair material with controllable injectability and self-setting properties prepared by introducing CSH into nHAC [16]. Meanwhile, Zheng et al. developed nanohydroxyapatite (nano-HA) reinforced collagen-alginate hydrogel (nHCA) that was prepared by the *in situ* synthesis of nano-HA in collagen gel followed by the addition of alginate and Ca^{2+}. The addition of alginate and nano-HA contributes to the increase in both mechanical and biological properties. This study may provide a valuable reference for the design of an appropriate composite scaffold for osteochondral tissue engineering [17].

By biomimetic strategy, many researchers have focused on the nHAC composites, which has shown a great promise in clinical applications because of their similar structural analogy to natural bone [18, 19]. The nHAC based scaffolds have now been successfully applied in the field of clinical applications, especially for the hard tissue repair [20].

In this study, we described a novel injectable material, composed of nHAC and alginate Alg. Then, human bone marrow stem cells (hBMSCs) were cultured on the nHAC/Alg material. The cell attachment, proliferation, and differentiation were evaluated and discussed.

2. Materials and Methods

2.1. Instruments and Equipment. In this study, centrifuge (produced by Heraeus company in Germany), inverted microscope (produced by Chongguang company in Chongqing), immunofluorescence microscope (produced by Lycra company in Germany), multiple tags immune analysis system (1420, WALLAC BLCTOR), CO_2 incubators (produced by Shellab company in Germany), Philips 525 M scanning electron microscope (produced by Philips company in Germany), and laser scanning confocal microscope (produced by Lycra company in Germany) were used to finish a series of testing experiments.

2.2. Reagent and Materials. The materials used in this study included 50 mL plastic bottle and 6, 24, 96-well culture plate (produced by Nunc company in Denmark), lymphocyte separation medium (percoll, 1.073 g/mL, produced by Huamei company in Shanghai), medium DMEM, heparin, L-ascorbic acid, dexamethasone Dex and sodium glycerolphosphate (produced by Gibco company in America), trypsin (produced by Sigma company in America), FBS (fetal bovine serum, produced by Sijiqing company in Hangzhou), Rhodamine (produced by molecular probe company in USA), Leica Microsystems Heidelberg (produced by GmbH company in Germany), acid soluble type I collagen gel (solid content: 1%, produced by Beijing Beihua Fine Chemicals Co.), trisodium phosphate and calcium sulfate (produced by Beijing Yihua Fine Chemicals Co.), alginate (produced by China National Biotec Group chemical reagent co.), and calcium carbonate (produced by Beijing Modern Oriental Fine Chemicals Co.).

2.3. Preparation of nHAC/Alg. Firstly, sodium alginate and sodium phosphate ($Na_3PO_4{\cdot}H_2O$) were mixed in deionized water and stirred for more than 20 minutes to obtain complete dissolution of the pale yellow aqueous solution of alginic acid. Then, calcium sulfate and deionized water according to the set ratio were mixed in a beaker and then stirred until no significant magnetic particles existed, followed by being put for more than 24 hours to remove the static electricity. Thirdly, nHAC powders were dissolved into deionized water and stirred completely until the material like bone paste was gotten, which were then mixed with the prepared alginic acid and stirred completely to get a kind of uniform mixture A, which were then mixed with calcium sulfate pulp to get uniform mixture B that was kept quietly for some time until the crosslinking took place completely inside material B. Many previous studies have shown that the alginate hydrogel is satisfactory carrier to prepare injectable biomaterials. [13, 21–25] At last, the materials will have exposure in the Co60 irradiation (2.5 Mrad) for disinfection and sterilization for further use.

2.4. The Separation of hBMSCs. Firstly, normal iliac bone marrow was extracted from healthy donors. Then, the bone marrow was centrifugated using three-gradient centrifugation with lymphocyte separation medium. The part above the white ring was taken over and put into DMEM medium which contains 10% fetal bovine serum medium and glutamine. Then the cell solution was put in T-50 culture bottle with cell amount of 3×10^5/mL, which was then incubated at 37°C and 5% CO_2. The first replacement of medium was done at 48 hours. Afterwards, the medium was changed every other day. When the cells attached to and grew into confluence on the bottom of bottle, 0.5% trypsin was used to digest the attached cells down.

2.5. The Qualitative Observation of the Osteogenic Differentiation of the hBMCSs. On the basis of the original medium, dexamethasone 10^{-8}/mol, β-glycerin sodium phosphate 10 mmol/L, and ascorbic acid 50 μg/mL were added as osteogenesis conditioned medium that was replaced every other day. After 3 weeks, the hBMSCs were observed under microscope.

2.6. The Culture of the hBMSCs on the nHAC/Alg Materials. The third generation of hBMSCs (2×10^4 cells/cm^2) was cultured on the nHAC/Alg material, incubated for four hours at 37°C and 5% CO_2. Then, the culture medium was added when the cells attached well to the materials. The culture medium was changed every 3 days. Culture plates washed by NaOH were used as blank control.

2.7. Inverted Microscope Observation. The cell growth situation on the culture plates, the cell induced situation, and the cell growth situation cultured on the material were observed, respectively, under the inverted microscope daily.

2.8. Scanning Electron Microscope Observation

2.8.1. The nHAC/Alg Materials. The nHAC/Alg materials were cut into 1-2 mm thick discs and fixed with 2% glutaraldehyde, followed by critical-point drying and gold-plating. Then, the material surface was observed with Philips 525 M scanning electron microscope.

2.8.2. The nHAC/Alg Materials Cultured with the Cells. The nHAC/Alg materials were cut into 1-2 mm thick discs. Then the cells were cultured on the materials for 3 and 7 days according to 2.6. And then, the samples were fixed with 2% glutaraldehyde, followed by critical-point drying and gold-plating. Then, the material surface cultured with the cells was observed with Philips 525 M scanning electron microscope.

2.9. Laser Scan Confocal Microscope (LSCM) Observation. The nHAC/Alg was cut into 1-2 mm thick discs, immerged in 1% Rhodamine solution for 5 minutes, immersed in distilled water for 30 minutes, dried, and disinfected by Co60 irradiation. The hBMSCs were dyed by DAPI (60 μm per unit) for half an hour, then washed for 3 times, and vaccinated on the materials which had been dyed by Rhodamine. The growth of cells which had been cultured for 2 days and 4 days on the material would be observed under the laser confocal microscope.

2.10. Determination of Cellular Activity with MTT. nHAC/Alg was cut into 1-2 mm thick discs and then fixed on 24-well culture plate for prewetting. Twelve wells were each set by a piece of material and another twelve wells were set as blank controls without material. The hBMSCs were cultured with 5×10^4/sample, incubated for four hours. Then 2 mL culture medium was added into each well. On the second, fourth, and sixth day, four wells would be selected to add 20 μL MTT (5 mg/mL), incubated for four hours and then stopped. The supernatant of each well was abandoned. Every well had an addition of 150 μL DMSO and was oscillated for 10 min to make crystal dissolve completely. Finally, the plate was read with a BIO-TEK automate microplate reader at 540 nm.

2.11. Determination of Alkaline Phosphatase Activity. nHAC/Alg was cut into 1-2 mm thick discs and then fixed on 24-well culture plate for prewetting. Twelve wells were each set by a piece of material and another twelve wells were set as blank controls without material. The hBMSCs (1×10^5/mL) were cultured on these samples and incubated for four hours. Then 2 mL culture medium containing heparin, ascorbic acid, dexamethasone, and glycerin sodium phosphate was added into each well. At 2, 4, and 6 days, the plate was read with a BIO-TEK automate microplate reader at 405 nm.

2.12. Statistical Methods. The data obtained will be analyzed with SPSS10.0 statistical software for statistical analysis. $P < 0.05$ indicated that there was a statistical difference.

3. Result

3.1. Inverted Microscope Observation

3.1.1. Observation of hBMSCs. After 24 hours of inoculation, there were still some floating blood cells with a large number of breed in bone marrow cell suspension which experienced three-step centrifugal (Figure 1(a)). A few hBMSCs whose cell appearance was fiber samples could be observed with the replacement of solution. After 48 hours' inoculation and first replacement of solution with removing the vast floating adherent cells, a small amount of hBMSCs had full stretch into long spindle cells (Figure 1(b)). After 72 hours' inoculation, hBMSCs cells began division and proliferation with a significant increase of volume (Figure 1(c)). After 7 days' inoculation with a further proliferation, a clear demarcation appeared between the nuclei pulp, nucleus located in the central, and single nucleolus in the majority. The shining cell body and strong refraction showed the cells with long spindle in good condition. These cells were close to each other to form early colony formation (Figure 1(d)). After 14 days, cells proliferated in colony growth and became mutual connection (Figure 1(e)). These monolayer cells represented with 0.25% trypsin digestion.

3.1.2. Observation of hBMSCs' Osteogenesis Induction. The morphology of the hBMSCs changed to short, square and fusiform shape after osteogenic induction (Figure 2). This result suggested that the hBMSCs can be induced into osteogenic cells.

3.1.3. Observation of the Proliferation of the hBMSCs on the Material. Because the material is opaque, the cells on the material surface were difficult to be observed under inverted microscope. The growth of the cells on the material could be observed through the pore when the material had been cut into 1 mm piece. On the first day, the cells adhered to the pore and the surroundings (Figure 3(a)). As the time went on, the cells on the material gradually attached to and proliferated well on the wall of the pore (Figure 3(b)).

3.2. Scanning Electron Microscope Observation. nHAC/Alg is a kind of porous framework material with random polygonal holes, 5–15 μm thick flat hole wall, and 100–300 μm aperture. The holes of the horizontal and vertical formed the connected structure (Figure 4(a)). On the third day of culture, the cells with extended pseudopodia adhered to the nHAC/Alg's surface and pores. A lot of visible microvilli on the cell surface showed the cells in a good condition (Figure 4(b)). On the 7th day, with the increase of cell number, there have been granular calcium crystals deposited on the cell surface. Cells are interconnected and the cell morphology preserved (Figure 4(c)).

3.3. LSCM Observation. The nHAC/Alg material that was dyed by Rhodamine appeared green and the hBMSCs that were dyed by DAPI appeared blue. The materials cultured with the cells, respectively, for 2 and 4 days were cut

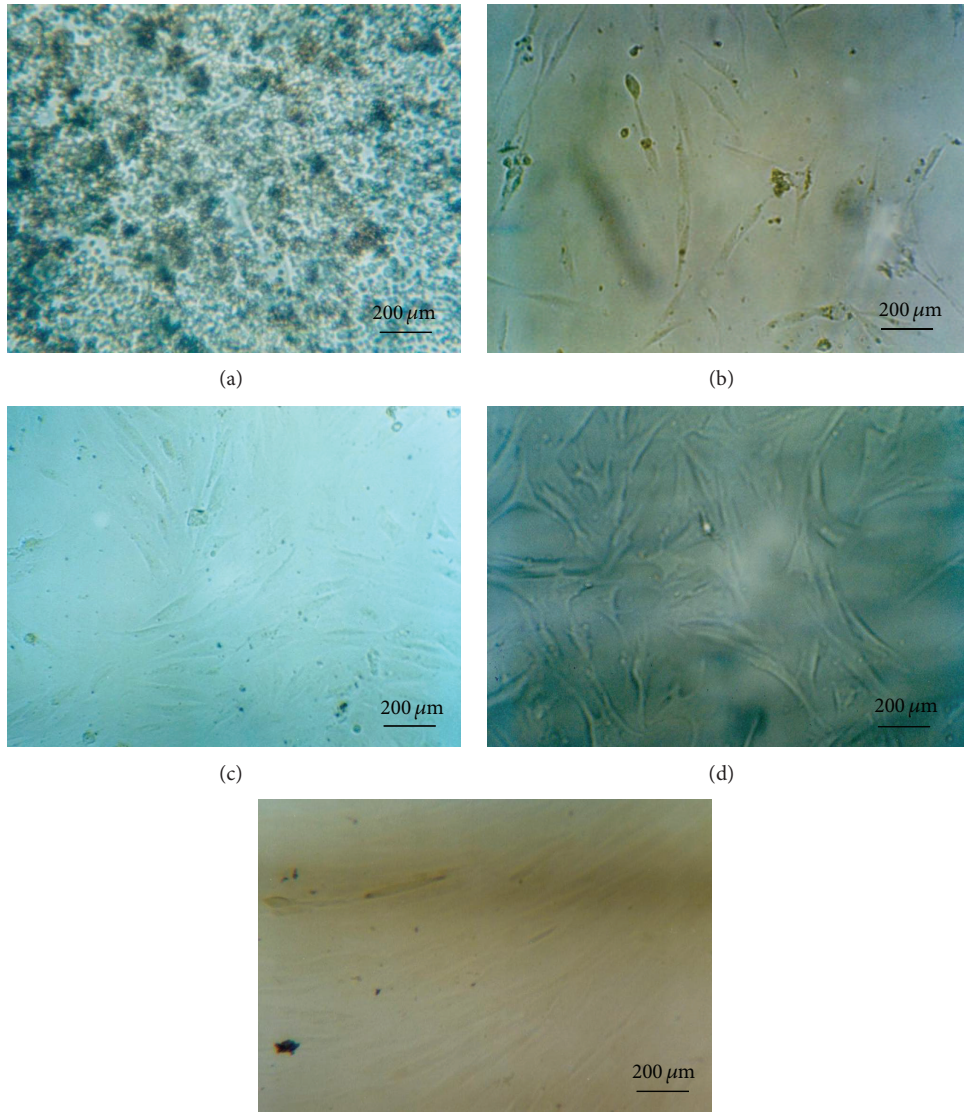


Figure 1: The cell morphology after the culture of (a) 24 hours; (b) 48 hours; (c) 72 hours; (d) 7 days; and (e) 14 days.

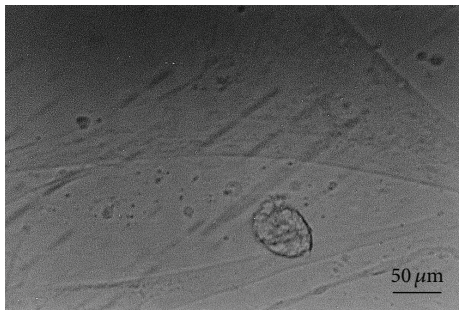
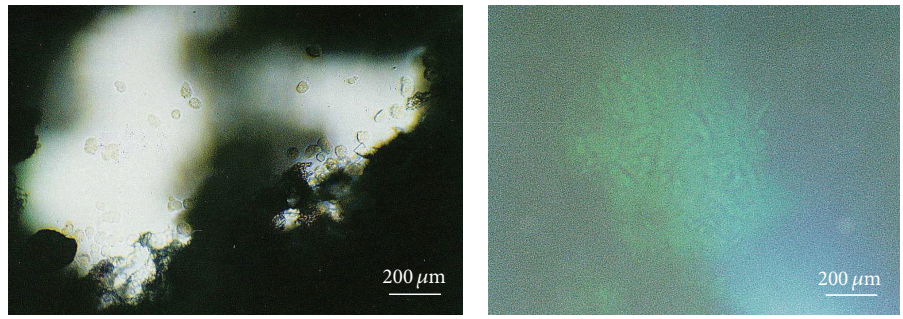


Figure 2: The cell morphology after osteogenic induction.

into thin pieces along the longitudinal direction to see the distribution of the cells inside the material. At day 2, dyed-blue hBMSCs attached better not only to the surface but also inside the pores of the dyed-green nHAC/Alg (longitudinal distance 19.5 μm) (Figure 5(a)). At day 4, the blue area has become larger because the cells attached to the materials have proliferated significantly (longitudinal distance 22.7 μm) (Figure 5(b)).

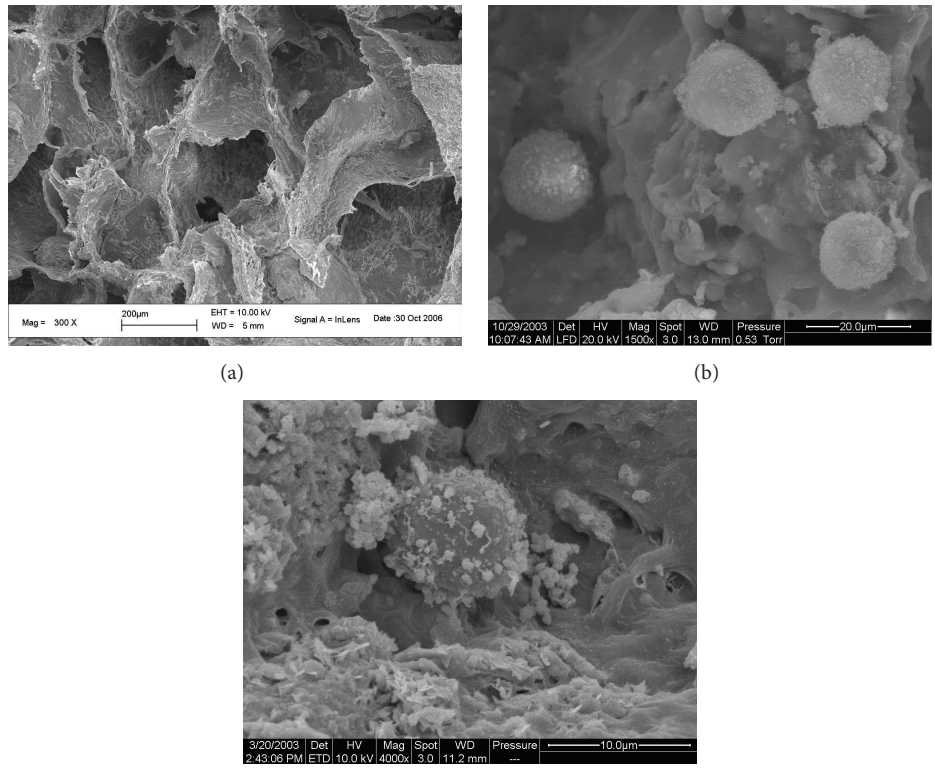
3.4. Determination of Cell Activity Determined by MTT Method. Figure 6 shows the 490 nm wavelength light absorption value of materials cultured for 2, 4, and 6 days. It was shown that there was no significant difference between the value of the cells cultured on the materials and that of control ($P < 0.05$) at each time point.

3.5. Determination of Alkaline Phosphatase Activity. Alkaline phosphatase activities of the cells cultured on nHAC/Alg materials and control at culture time of 2, 4, and 6 days



(a) (b)

Figure 3: The situation of the cell attachment and proliferation on the wall of pores of the nHAC/Alg materials after the culture of (a) 1 day and (b) 4 days.



(a) (b)

(c)

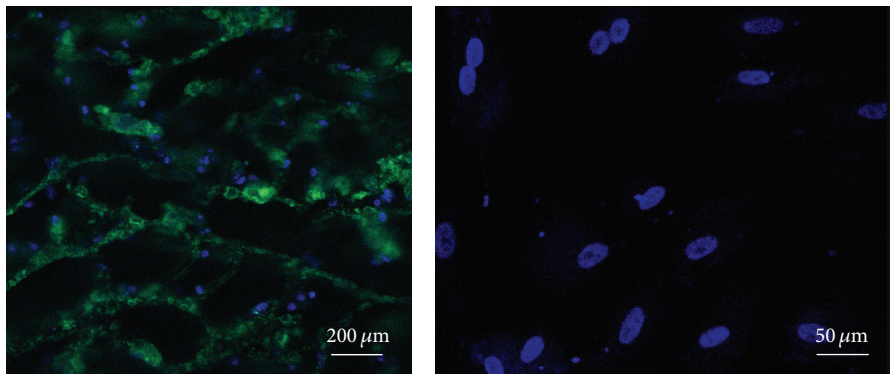
Figure 4: Scanning electron microscopes of (a) the nHAC/Alg surface; (b) the nHAC/Alg surface cultured with the hBMSCs cells on the third day; and (c) the nHAC/Alg surface cultured with the hBMSCs cells on the 7th day.

were shown in Figure 7. The values of the control did not change significantly ($P > 0.05$) during the test period. At each time point, the value of the cells cultured on the materials was significantly higher than that of control. During the test period, the value of the cells cultured on the materials significantly increased ($P < 0.05$).

4. Discussion

How to evaluate the biocompatibility of nHAC/Alg is the key point to judge whether it is suitable to be bone substitute. The composite of nHAC/Alg and hBMSCs observed under inverted phase contrast microscope showed that the cell growth on the material had no abnormalities. But that is just one aspect of the biocompatibility. Whether the cultured cells had normal physiological function is the most important criterion to evaluate the biocompatibility of biomaterials.

The principle of LSCM was installing a laser scanning device on the fluorescence microscope imaging, using the computer for image processing and ultraviolet or visible fluorescence probe to get the fluorescence image of cells or tissue's internal fine structures. LSCM can not only reveal



(a) (b)

Figure 5: LSCM of the nHAC/Alg cultured with the hBMSCs cells at day 2 and (b) the nHAC/Alg cultured with the hBMSCs cells at day 4.

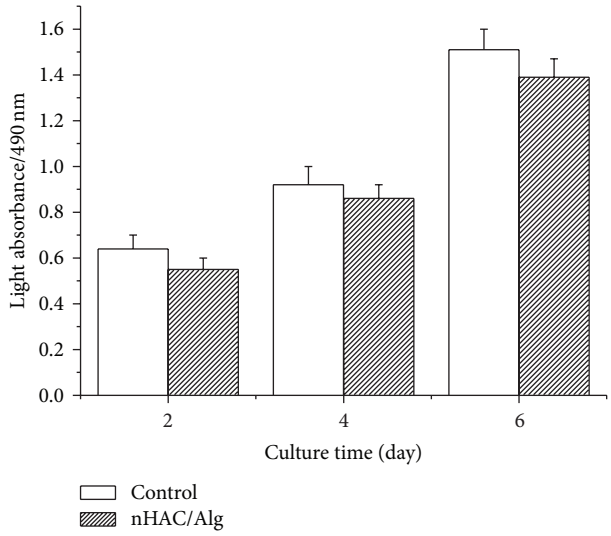


Figure 6: The results of MTT at discrete time of 2, 4, and 6 days.

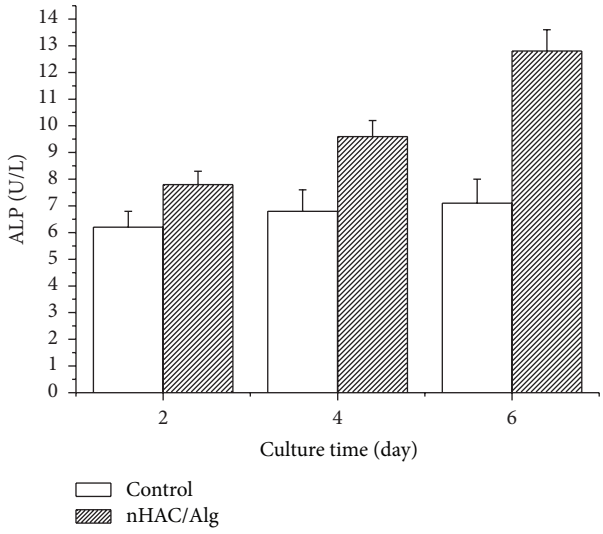


Figure 7: Alkaline phosphatase activity of the cells cultured on nHAC/Alg materials and control at discrete time of 2, 4, and 6 days.

the cell's internal structure and provide the cell length, width, thickness, fault area, and cell volume, but also give a three-dimensional concept. Without damaging cells, LSCM can observe and measure living cells and even living tissue. So, the sample can still be used in other studies [26, 27]. The shape of the living cells on materials can be directly observed by LSCM, which can collect images through the mark on the material or fluorescence substance of the cells [28]. The cells and material carrier were, respectively, labeled by fluorescent dye of different colors and then were complex cultured. It was shown in this study that dyed-blue hBMSCs attached better not only to the surface but also inside the pores of the nHAC/Alg. It was also shown by SEM that the cells adhered to the nHAC/Alg's surface and walls of pores with extended pseudopodia.

Recently, MTT was usually used to test the toxic effects of materials on cells. The principle is that succinic dehydrogenase of living cells mitochondria can make exogenous tetrazolium salts reduction into difficult soluble violet crystal which deposited in cells while dead cells cannot. Dimethyl sulfoxide can dissolve the crystal and indirectly reflect the number of living cells through the determination of light absorption value [29]. The experiment results showed that there was no significant difference between the light absorption value of the cells cultured on the nHAC/Alg materials and that of control ($P < 0.05$) at each time point, which suggested that the materials could support the cell proliferation well.

ALP is an early marker of osteoblast differentiation and is one of the symbols of mature osteoblast differentiation [30]. Many experimenters used ALP to evaluate the situation of varieties of biological experiments and the level of the osteogenic differentiation of cells [31, 32]. The results of this study showed that, at each time point, the value of the cells cultured on the materials was significantly higher than that of control. During the test period, the value of the cells cultured on the materials significantly increased ($P < 0.05$), which suggested the nHAC/Alg materials could promote the differentiation of the hBMSCs into osteogenic cells.

Our findings could make connection with the work of Han et al. [33] who reported the preparation of the calcium

silicate/alginate (CS/Alg) composites and the evaluations of its behavior as bioactive injectable hydrogels.

Above all, the hBMSCs attached better not only to the surface but also inside the pores of the nHAC/Alg material. The material could support the cell proliferation well. Most importantly, the nHAC/Alg materials could promote the differentiation of the hBMSCs into osteogenic cells, which suggested that this nanoscaled injectable material should be a satisfactory candidate as bone repair material.

5. Conclusion

In this study, we have prepared a kind of novel nanoscaled injectable bone repair materials (nHAC/Alg). The nHAC/Alg not only had no negative effect on cellular functions but also promotes cell proliferation and differentiation into osteogenic cells, which suggests that the nanoscaled injectable bone repair material has good clinical application prospects for bone repair.

Conflict of Interests

The authors have no conflict of interests.

Acknowledgment

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