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Original Article

Design and evaluation of poly (lactic-co-glyclic acid)/poly (vinyl alcohol)/nano-hydroxyapatite hydrogels for cartilage tissue engineering in vitro

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Abstract: A major challenge in cartilage tissue engineering is the scaffold. Poly (vinyl alcohol) (PVA) hydrogel has great potential because of its structure and material properties. But it still has some deficiencies, such as its insufficient mechanical property and poor biocompatibility. Nano-hydroxyapatite (nano-HA), as a bone repair material, could improve the mechanical property of scaffolds, while poly (lactic-co-glyclic acid) (PLGA), a degradable polymer, could improve the biocompatibility. So far, their synergic effects on PVA hydrogels had not been studied yet. In this paper, through solvent extraction/evaporation technique and freeze-thaw cycling method, the PLGA/PVA/nano-HA composite hydrogels were prepared. It was found that the morphological characterization of the composites could be changed by altering the content of raw materials. When the concentration of PVA was on a scale from 5 wt% to 15 wt%, the moisture content of the composites was in the ranged 74-84%, average pore size 80-118 μm, porosity 57-64%. Then the human mesenchymal stem cells (MSCs) were seeded in these composites to assess the biocompatibility and practicability in vitro by comparison to PVA/nano-HA hydrogels. The results of cell culture test showed that human MSCs were able to attach, grow and proliferate well in the composites. In addition, the composites could promote the proliferation and differentiation toward chondrocytes in vitro.

Keywords: Poly (lactic-co-glyclic acid), poly (vinyl alcohol), nano-hydroxyapatite, tissue engineering, human mesenchymal stem cells, cartilage

Introduction

Cartilage tissue is difficult to regenerate due to its poor blood supply, special biomechanics and complex structure [1, 2]. It is generally believed that the damage can be partially or completely repaired when the diameter of the damage is less than 3 mm, and that the self-repair ability would be obviously limited when the diameter of the damage is more than 4 mm [3, 4]. Although various tissue engineering approaches to repair cartilage defects have been investigated, there is still not a perfect approach [5], and the main challenge is the scaffolds [6, 7]. The three-dimensional scaffolds are explored for the filling of cartilage defects, which including natural and synthetic origin materials [8, 9]. The former have great biocompatibility, but poor mechanical properties and immunological rejection, while the latter have various mechanical properties [10].

PVA hydrogel, as a non-degradable synthetic polymer, has great potential for development in cartilage tissue engineering, because its structure and material properties share similarities with natural cartilage, besides, the synthetic polymer shows greater control over the composition and structure than natural materials [11-14]. But the PVA hydrogel still has some problems, such as its insufficient mechanical property and porosity, small pore size and poor cell adhesion ability, which limit the further fundamental research and clinical application [15, 16]. In order to overcome these drawbacks, several scientific literatures have shown that some materials such as type II collagen and PLGA are often used to improve the cell adhesion ability of the hydrogel, nano-HA to increase the biomechanics and bioactivities [17-19]. Furthermore, they have confirmed that the composite hydrogel has excellent biomechanics and tribological properties when the mass frac-

tion of nano-nano-HA is 6 wt% [20, 21], and it has great biocompatibility and suitable porosity when the mass fraction of PLGA is 30 wt% [18]. We have tried to construct a novel PVA composite hydrogel that has sufficient biocompatibility and appropriate porosity and pore size. Through blending PLGA and nano-HA to improve the biocompatibility and biomechanics, we have shown that we could create super-porous and biocompatible hydrogels.

Chondrocytes, as alternative seed cells in cartilage tissue engineering, have some limitations, such as their poor sources and dedifferentiation, which restrict their application [22, 23]. MSCs have great potential in cartilage tissue engineering for their great chondrogenic differentiation potential and rich source [24-26]. And the nature of materials and physicochemical properties of scaffolds affect the ability of chondrogenic differentiation of MSCs and the secretion of extracellular matrix [27, 28].

In this study, the composite hydrogels, formed by PVA, PLGA and nano-HA, were physically cross-linked by solvent extraction/evaporation technique and freeze-thaw cycling method. Changing the quantity of raw material could alter the physicochemical properties of the hydrogels. According to the morphological characterization of a series of scaffolds, this study chose the superior composites for further cell experiments. Finally, our finding evaluated the efficiency of seeding human MSCs and the practicability of using PLGA and nano-HA to improve chondrogenesis in the composites. We hypothesize that these novel composite hydrogels would be suitable for adhesion of human MSCs and the expression of cartilage in vitro.

Materials and methods

Composite hydrogels preparation

The PLAG/PVA/nano-HA composite scaffolds were fabricated using solvent extraction/evaporation technique and freeze-thaw cycling method [29, 30]. Firstly, a certain dose of PVA (99+% hydrolyzed, Mw89000-98000, sigma, USA) and nano-HA (sigma, USA) were incorporated into some double distilled water. The mixtures were heated to 90°C for 90 minutes in water bath with the thermostatic magnetic mixer stirring. A certain dose of PLGA (lactide: glycolide 50:50, ester terminated, Mw38000-54000, sigma, USA) was dissolved through ultrasonic stirring in dichloromethane, which was served as milk-white primary emulsion. Then, the primary emulsion was added to the PVA-nano-HA mixture. The PLGA/PVA/nano-HA solution was stirred by the thermostatic magnetic mixer so as to evaporate the dichloromethane. And then, the solution was carefully injected into a mould. Finally, the mould was freezing for 21 hours at -20°C and let it thaw for 3 hours at room temperature, the freeze-thaw cycle was repeated for 5 times so as to increase the density of cross linking.

As mentioned above, the composites, with 30 wt% PLGA and 5 wt% nano-HA, were divided into six groups according to the mass fraction of PVA (group A: 5 wt%; group B: 10 wt%; group C: 15 wt%; group D: 20 wt%; group E: 25 wt%; group F: 30 wt%).

Morphological characterization of hydrogels

The PLAG/PVA/nano-HA composite scaffolds and each raw material were detected at room temperature through Raman spectra analysis (LabRAM HR800, Jobin-Yvon, France). The laser wavelengths of the helium neon laser was 633 nm, the power 25 mW, the grating 1800 gr/mm, the resolution 0.3 cm^{-1}, and the scanning time 10 seconds.

The moisture content of the composites was measured by calculating the difference in weight between dry and swollen composites. The PLAG/PVA/nano-HA composite scaffolds were dried to constant weight, recorded as W_d. When the dry hydrogels reached swelling equilibrium in saline, we recorded the swollen weight as W_s. So the degree of swelling was measured as follow:

Degree of Swelling (%) = $(W_s-W_d)/W_d \times 100\%$ formula (1)

Each value was average from three independent measurements.

Since the hydrogel scaffolds would play a role in water gel state, microscopic morphology should be detected when the scaffolds are in water-bearing and moist state. For this reason, environmental scanning electron microscope (ESEM) (Quanta-200, FEI, Netherlands) was used to analyze the microscopic morphology.

The scaffolds were cut into 5×5×5 mm^3 cubes, and the moist cubes were fixed on the electronic conductive adhesive. Under the condition of low vacuum mode (99.75 Pa), high pressure (20, kV), and beam spot, the microscopic morphology of each sample was observed.

With the help of the photo in microscope, the determination of pore size and PLGA particle diameter were measured by Image J software (version: 1.48 v, National Institutes of Health, USA). Three photos in each group were selected, and each photo was measured three times.

The porosity of hydrogel scaffolds was calculated by the Archimedes principle. The sample was freeze-dried by freeze dryer (FD-1D-50, Sjialab, China), the weight and volume of which was recorded as M_1 and V respectively. The weight of a pycnometer filled with alcohol was recorded as M_2. Then the sample was cut into pieces and immersed in alcohol so that the pores were full of alcohol with the help of a vacuum pump. The spilled alcohol was cleaned up, and then the weight of the pycnometer filled with alcohol and sample was recorded as M_3. The actual volume (V_m) of the sample was measured as $V_m = (M_1+M_2-M_3)/\rho$, where ρ is the density of alcohol. The porosity was calculated by the formula:

Porosity (%) = $(V-V_m)/V\times100\%$ formula (2)

Each value was average from three independent measurements.

Practicability of hydrogels in cartilage tissue engineering

Based on morphological characters we have detected, we selected these groups that the mass fraction of PVA was 15 wt%, 10 wt% or 5 wt%, PLGA 30 wt%, and nano-HA 5 wt%, as experimental subjects in vitro, and the scaffolds in the control group were only composed of nano-HA 5 wt% and PVA 20 wt% [21]. Thirty subjects in each group were in experiment. One third of that was used in detecting cellular adhesion and proliferation ability, and the rest in chondrogenesis of human MSCs.

The human MSCs (NO: 130407F01, Cyagen, USA) have been confirmed by cell morphologies, cell surface markers and differentiation experiments. In summary, the steps for human MSCs amplification were as follow.

1. Preparation of human MSCs growth medium: mixed Glutamine solution (5 ml), Penicillin-Streptomycin solution (5 ml) and Mesenchymal Stem Cell-Qualified Fetal Bovine Serum (50 ml) with Human Mesenchymal Stem Cell Basal Medium (440 ml), and pre-warmed to 37°C.

2. Thawed out human MSCs into T25 flask and incubated with the medium above at 37°C in a 5% CO_2 humidified incubator.

3. Collected sub-culture cells when cells reached 80-90% confluence and adjusted cell densities of the cell suspension to be 1×10^6/ml.

Specific steps were as follow.

1. freeze-dried the hydrogel scaffolds (5×5×5 mm^3) with lyophilized (VFD-1000, Biocool, China), next sterilized with dichloromethane, and then rinsed with PBS solution repeatedly, finally, transferred the scaffolds to 24 hole cell culture plate.

2. A certain amount of cell suspension (100 μl) dropped on the scaffolds, and the complexes were incubated at 37°C in a 5% CO_2 humidified for 2 h, and then carefully added 2-3 ml of fresh human MSCs growth medium (pre-warmed to 37°C) to each hole, incubated at 37°C in a 5% CO_2 humidified incubator for 1 day.

3. Preparation of human MSCs chondrogenic differentiation medium: the incomplete chondrogenic differentiation medium was composed of Human Mesenchymal Stem Cell Chondrogenic Differentiation Basal Medium (97 ml), Dexamethasone (10 μL), Ascorbate (300 μL), ITS+Supplement (1 ml), Sodium Pyruvate (100 μL) and Proline (100 μL). 10 μL of TGF-β3 would convert 1 mL of incomplete medium into complete chondrogenic medium.

4. The next day, incubated medium should be changed into human MSCs chondrogenic differentiation medium.

5. Changed the medium every 2-3 days thereafter and observed the morphological changes of cells by inverted microscope (Leica, Germany) everyday.

24 h after plating, five composites were exposed to PBS solution to elute cells. By cell counting, the adherence rate was determined

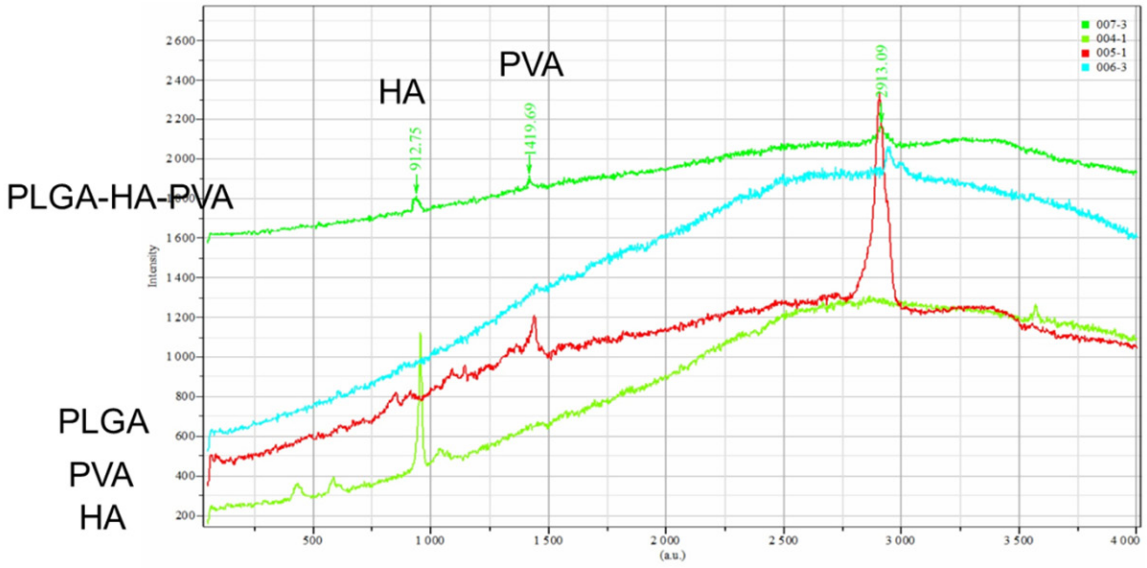

Figure 1. Raman spectra analysis of the raw materials and composites.

by the percentage of total adhesive cell number.

Three days after cell culture, MTT assay was used to evaluate cellular proliferation. A blank control group containing only medium and cells was included in this step. According to manufacturer's instructions: Firstly, after the addition of 20 μL MTT (5 mg/ml) for 4 h, the supernate was discarded. Then, with oscillations, 50 μL dimethyl sulfoxide was added to each hole. Finally, at a wavelength of 490 nanometers, the relative proliferation was assessed by enzyme-linked immunometric meter (Sunrise, Switzerland).

Chondrogenesis of MSCs in hydrogels

After 3 weeks of culture, five composites including scaffolds and cells were used for hematoxylin-eosin (HE) staining. The samples processed for HE staining needed to go through these steps.

1. Fixed by 4% paraformaldehyde for 24 h, then embedded by paraffin.

2. Cut the complexes into 5 μm-thick sections, and put sections under the condition of 60°C for 45 min.

3. Section dewax was done twice by xylene for 10 min, next the slices were dehydrated by 100% alcohol, 95% alcohol, 85% alcohol and 75% alcohol for 2 min in turn.

4. Firstly dyed with hematoxylin semen for 5 min, and then with eosin solution for 5 min.

5. After dehydration again with alcohol, dewax was used to make sections more transparent.

6. Phosphate buffer saline (PBS) was used to wash sections between each step.

7. After fixation with neutral resins, photomicrographs were taken with a microscope (Leica, Germany).

Quantitative analysis of collagen type II (COL2) and glycosaminoglycan (GAG) was measured by Western blotting after 7, 14 or 21 days in culture. Following manufacturer's instructions, first extracted protein from the complexes, next performed gel electrophoresis, then transferred protein to nitro cellulose, after that conducted immunization response and coloration, finally the gray level distribution was analyzed by Quality one software, and the relative expression of blotted protein was measured by comparing the gray level.

Statistical analysis

All values are reported as mean ± standard deviation (SD). When obeying normal distribution and homogeneity of variance, statistical

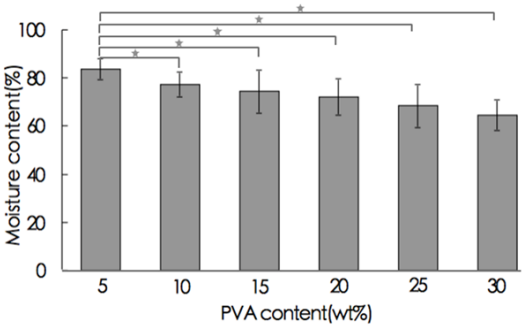

Figure 2. Swelling properties of the composites (*P<0.05).

analysis of differences between groups were evaluated using one-way ANOVA, and the pairwise comparison among the means was done by LSD method. In addition, Tamhane's T2 test was used for the unequal variances. With the use of SPSS19.0 statistical software, statistical significance was defined as P<0.05.

Result and discussion

Raman spectra analysis

According to spectra analysis (**Figure 1**), the location of a peak corresponded to a component. The peaks of nano-HA, PVA and PLGA were located in 912.75 cm^{-1}, 1419.69 cm^{-1} or 2910 cm^{-1}, and 2950 cm^{-1} respectively. In the analysis of PLGA/PVA/nano-HA composite scaffolds, the characteristic peaks of each substance were appeared, and there was no other peak, and due to the overlapping of peaks, the peaks of PVA and PLGA were not fully embodied (**Figure 1**).

The results showed that the PLGA/PVA/nano-HA composites prepared by solvent extraction/evaporation technique and freeze-thaw cycling methods have no visible evidence of impurity. During the entire making process of composites, not any other chemicals are required beyond dichloromethane, a volatile liquid. By stirring constantly, dichloromethane was volatileout.

Swelling properties

As shown (**Figure 2**), the swelling properties could be compared among there groups.

Data showed that the moisture content of each group was more than 60%. Through statistical analysis, the moisture content of group A was greater than the other groups. In this study, the PVA hydrogel was physically cross-linked, in the form of entanglements or hydroxyls among molecular and secondary forces, such as hydrogen bonding, Van der Waals interactions, by freeze-thaw method rather than chemical crosslink, then H_2O and other small molecules were easily bonded with hydrophilic -OH group [31, 32]. Through repeating freeze-thaw cycle, the overall crystallinity was increased and the volume of amorphous region filling with water was decreased, then the microstructure of composites was changed into a fibrillar network. As the concentration of PVA increased, the volume of amorphous region decreased, which leaded to decreased swelling properties.

ESEM detection

By analyzing the pictures (**Figure 3**), we could see obvious porous structures in the PLGA/PVA/nano-HA scaffolds, which mainly constituted by PVA. In addition, the PLGA particles attached to the walls of the pores, and nano-HA distributed evenly inside the scaffolds.

In the process of freeze-thaw, molecular of PVA was redistributed and gathered to create the polymer-rich regions, which were separated by watery polymer-poor regions [30]. And the watery regions were the sites of pores. After repeating freeze-thaw cycle and addition of nano-HA, the pore structures were increasingly stable [30], which facilitated the adherence of cells. As bio-inert polymers, the hydrophilic PVA hydrogels went against cell adhesion and growth [33]. In contrast, the characteristic of hydrophobic PLGA particles facilitated the integration with surrounding cartilage [18, 34]. In this study, the PLGA particles were prepared through solvent extraction/evaporation technique. We evaluated the benefits of adding the micro particles to the hydrogels by the following cell experiments. There are reports that nano-HA has been used to improve the bioactivities and mechanical properties of the composites [20, 35]. Because of its high dispersibility and structure stability, nano-HA was distributed evenly inside the scaffolds. It is reported that the nano-HA/PVA composites has excellent biomechanics when the concentration of nano-HA is 6 wt% [20, 21]. The limitation of this paper was that the mechanical properties of the composites were not done yet.

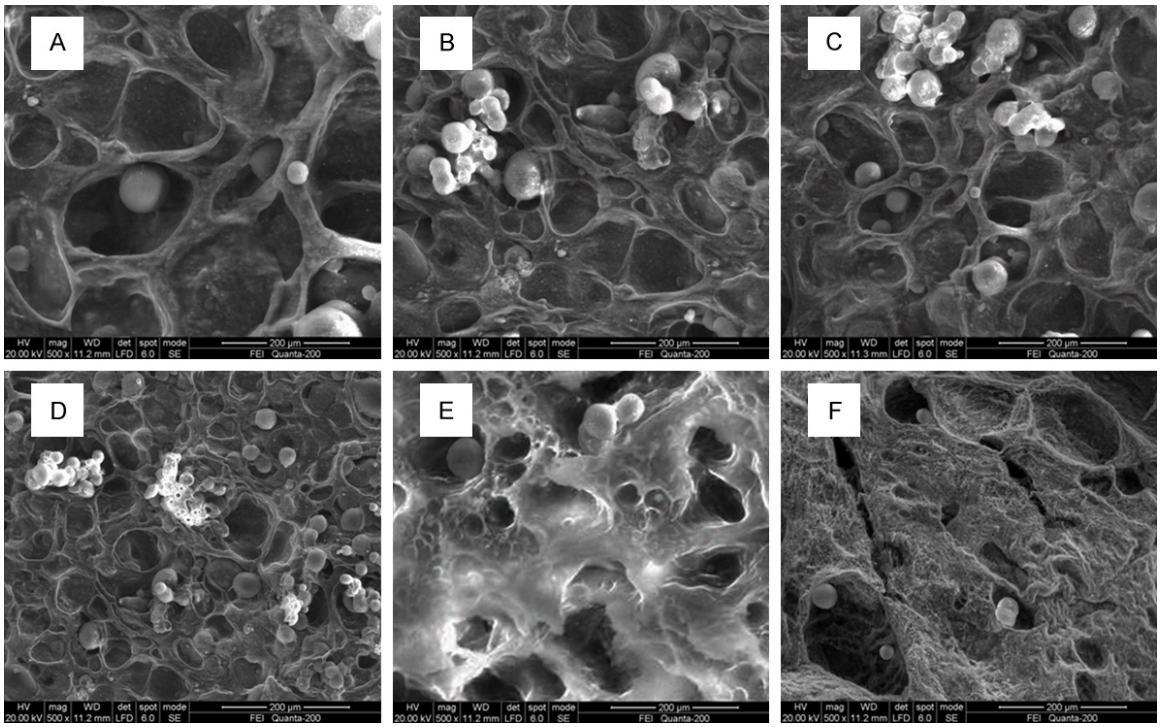

Figure 3. ESEM images of PLGA/PVA/nano-HA composite hydrogels. Scale bars: 200 μm. A: 5 wt% PVA+30 wt% PLGA+5 wt% nano-HA; B: 10 wt% PVA+30 wt% PLGA+5 wt% nano-HA; C: 15 wt% PVA+30 wt% PLGA+5 wt% nano-HA; D: 20 wt% PVA+30 wt% PLGA+5 wt% nano-HA; E: 25 wt% PVA+30 wt% PLGA+5 wt% nano-HA; F: 30 wt% PVA+30 wt% PLGA+5 wt% nano-HA.

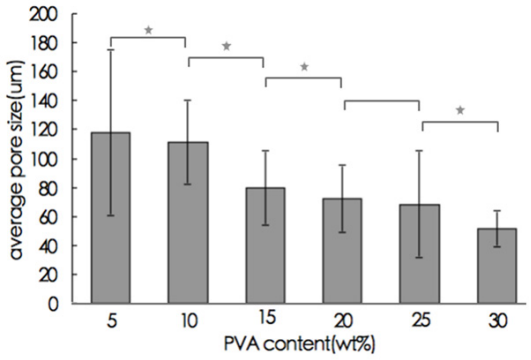

Figure 4. Average pore size, determined using ESEM and Image J software (*P<0.05).

The results of pore size and PLGA particle diameter were showed in **Figures 4** and **5**.

With the increasing of PVA content, the average pore size of scaffolds was decreased, presenting A>B>C>D and E>F (P<0.05), and there was no statistical difference (P = 0.15) between group D and group E. As the concentration of PVA increased, the proportion of polymer-rich regions increased while polymer-poor regions decreased [30, 32], which leaded to the formation of smaller pores. In our research, the average pore size of group A was 117.74 μm, group B 113.30 μm, and group C 79.78 μm. It was known that pore size was a very important parameter of scaffolds, which should be large enough to benefit cell migration and permeability of nutrient while small enough to provide sufficient areas for cell attachment [36-38], meanwhile the optimal pore size of a scaffold was inconclusive, and in response to cell types [39]. Consequently, in follow-up experiments, we wanted to explore more detail about what size scaffolds were appropriate to the growth of cells.

With the increasing of PVA content, the PLGA particle diameter changed in the trend of small, but it was not statistical difference (P>0.05). In the future, loaded with cytokines, we assured that the PLGA particles might be a promising delivery system in situ.

Determination of porosity

The results of porosity were showed in **Figure 6**.

With the increasing of PVA content, the porosity of scaffolds was decreased, presenting A>B, C>D and E>F (P<0.05), besides there was no

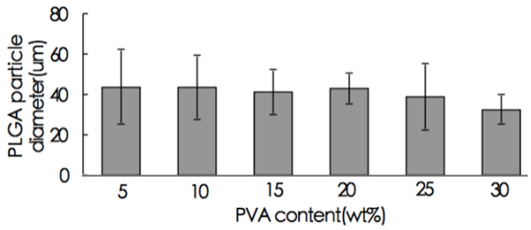

Figure 5. PLGA particle determination, determined using ESEM and Image J software.

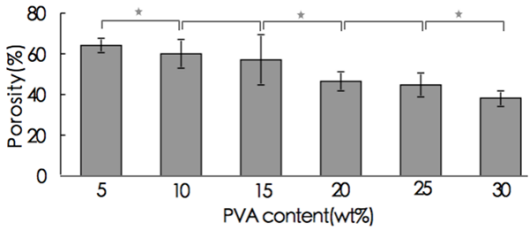

Figure 6. Porosity of PLGA/PVA/nano-HA composite hydrogels (*P<0.05).

statistical difference between group B and group C (P = 1.64), and between group D and group E (P = 0.64). Results showed that when the concentration of PVA was less than 15 wt%, the porosity of composites was between 57% and 64%. As reported, a high porosity represented large area/volume ratios, which facilitated cell adhesion, while porosity and mechanical properties were often conflicting [39]. In our study, the relatively superior scaffolds, lying in the first three groups, were used for further cell experiment in vitro. Honestly, the porosity was inadequate comparing with other papers [40], the reason might be that the addition of PLGA particles occupied the position of pores. With the degradation of PLGA, the porosity would gradually increase.

Cellular adhesion ability and proliferation

The morphology and growth feature of human MSCs were as follow (**Figure 7**).

On the first day after resuscitation, the cultured cells grew against the wall of flask, with spindle-shaped morphology; and the number of cells increased gradually; on the eighth day, cells were spindle shaped and whorled or parallel along longitudinal axis.

The results of cellular adhesion ability were showed in **Figure 8**.

The adhesion ability in the experimental groups was significantly stronger than that in the control one (P<0.05), while there was no statistical difference between the three experimental groups (P>0.05). This study found that the addition of PLGA dramatically favored the adhesion of human MSCs. As mentioned above, the hydrophobic PLGA particles and nano-HA facilitated the integration with surrounding cartilage and improved the porosity of scaffolds [18, 34, 41]. Through the combined effort of PLGA and nano-HA, the cellular attachment ability significantly increased. Since the mount of PLGA and nano-HA were identical, there was almost no difference among the three experimental groups.

The results of cellular proliferation were showed in **Figure 9**.

The proliferation in the experimental groups was stronger than that in the control one (P<0.05), further, the proliferation of the latter was stronger than that of the blank control one (P<0.05), in addition, there were no statistical difference between the three experimental groups (P>0.05). Some researchers have shown that the morphology and mechanical property of scaffolds, such as elastic modulus, affected how the seeded cells behave [42-44]. The results showed that the existence of these scaffolds benefited the proliferation of cells; furthermore the PLGA/PVA/nano-HA hydrogels were better than the PVA/nano-HA hydrogels. The reason for this phenomenon might be that the elastic modulus in the PLGA/PVA/nano-HA hydrogels was more suitable for proliferation than that in the PVA/nano-HA hydrogels. Accordingly, we might think that the increase of pore size and porosity helped to improve cellular proliferation.

The results of these tests led us to the conclusion that the addition of PLGA micro particles favored the adhesion and proliferation of seeded human MSCs.

Chondrogenesis of MSCs in hydrogels

HE staining results showed that plenty cells were observed in the pores of scaffolds (**Figure 10**).

The results of HE staining demonstrated that the cells proliferated well and secreted extracellular matrix, mimicking chondroid tissue. In

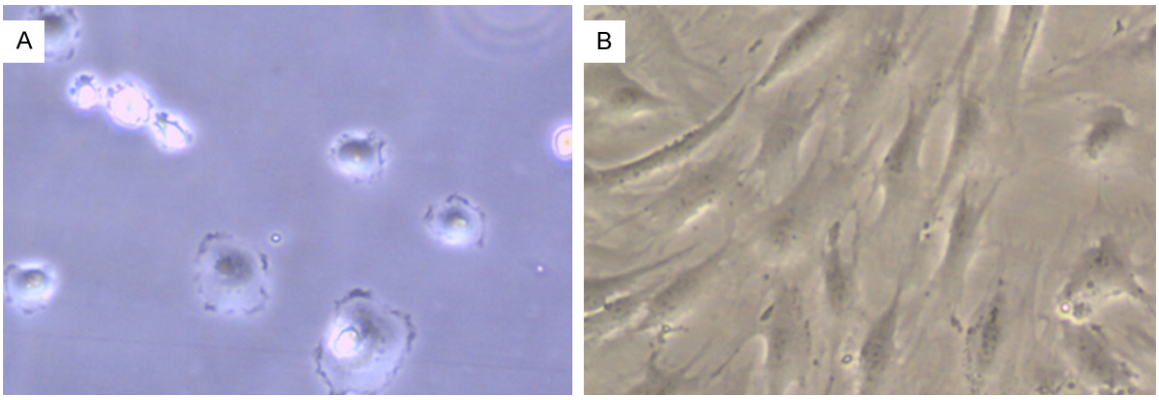

Figure 7. Morphological and growth feature of human MSCs by inverted microscope, magnification ×400. A: 2 hours after resuscitation B: 8 days of culture.

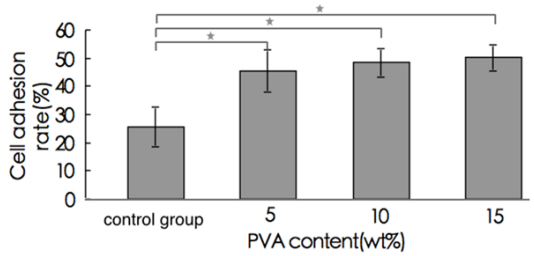

Figure 8. Cell adhesion rate of PLGA/PVA/nano-HA composite hydrogels, the scaffolds in the control group were only composed of nano-HA 5 wt% and PVA 20 wt% (*P<0.05).

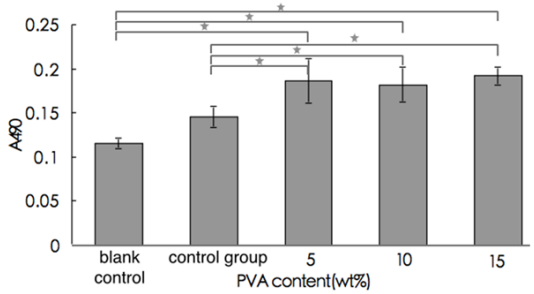

Figure 9. Relative cellular proliferation of PLGA/PVA/nano-HA composite hydrogels, the blank control group contained only medium and human MSCs (*P<0.05).

addition, the amount of matrix in experimental group was more than that in control group.

Results of Western blotting showed that the expression of COL2 and GAG could be detected in experimental and control group (**Figures 11** and **12**).

Main results were as follow: (1) The relative expression of COL2 and GAG increased over time (P<0.05); (2) When co-cultured for 21 days, the expression of COL2 and GAG in experimental group was higher than control group (P<0.05), meanwhile the expression in group C was higher than the other two groups (P<0.05), and the group B higher than group A (P<0.05).

This finding assured that the PLGA/PVA/nano-HA hydrogels, compared with PVA/nano-HA hydrogels, did better in improving the chondrogenic differentiation of human MSCs. The success of this novel composite was related to the addition of PLGA micro particles, which improved the biocompatibility of scaffolds. Meanwhile the amount of COL2 and GAG was higher in group C than that in the other two experimental groups. According to the above, the pore size and porosity in-group C was the least among the experimental groups. So we concluded that the chondrogenic differentiation of human MSCs could not be improved just by increasing the pore size and porosity. It is reported that the elastic modulus of scaffolds affects the differentiation of stem cells [45-47]. So we assured that the elastic modulus of the relatively small pore size and porosity might be more appropriate for differentiation. But more research is needed to explore the most suitable elastic modulus of this composite.

Conclusions

The morphological characterizations of this novel PLAG/PVA/nano-HA composite scaffolds, fabricating by solvent extraction/evaporation technique and freeze-thaw cycling method were changed in relation to PVA content. When PVA content was among 5-15 wt%, the pore size and porosity of the composites were appropri-

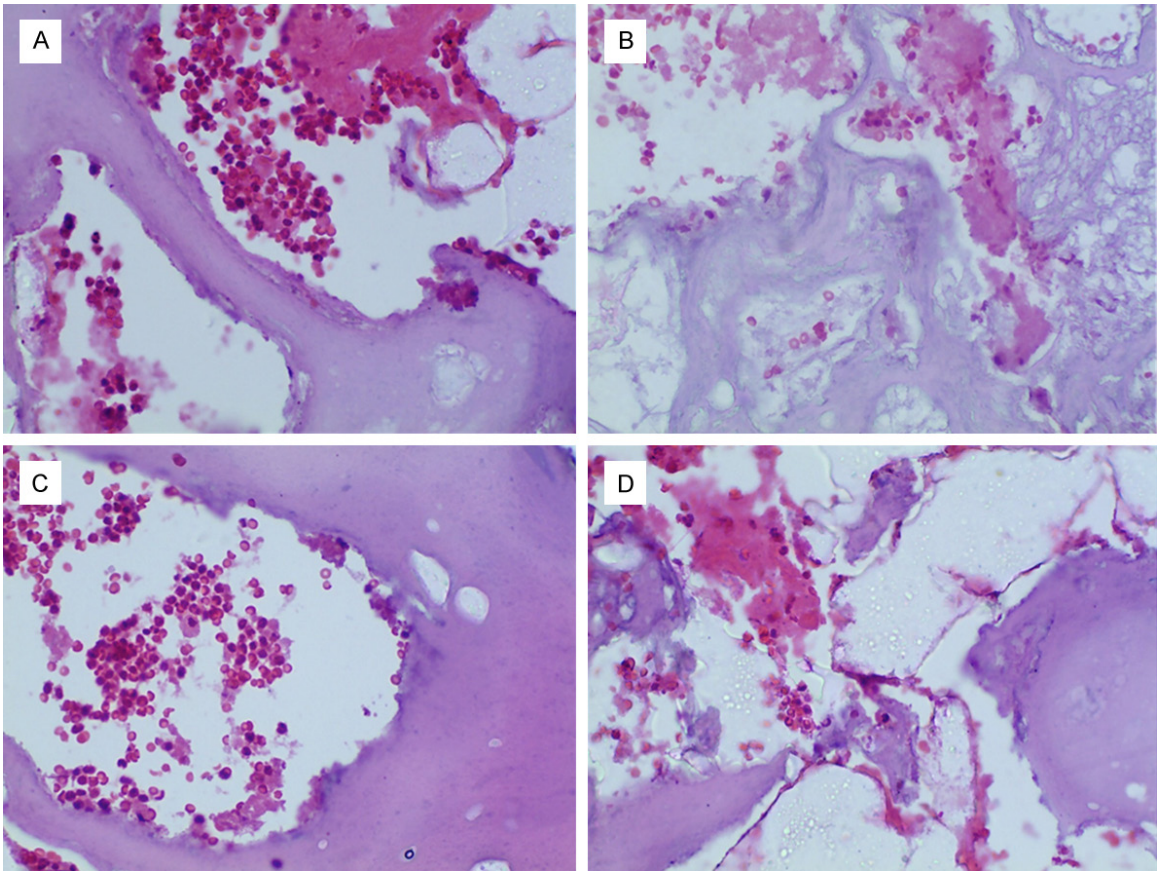

Figure 10. HE staining of the composites seeding human MSCs for 21 days, magnification ×100.

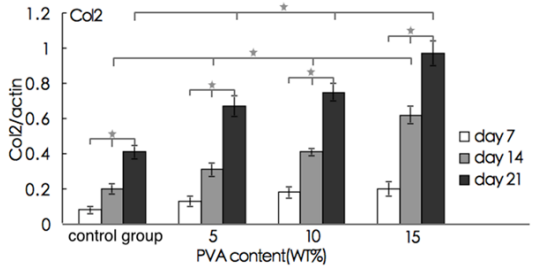

Figure 11. Relative expression of COL2 in scaffolds (*P<0.05).

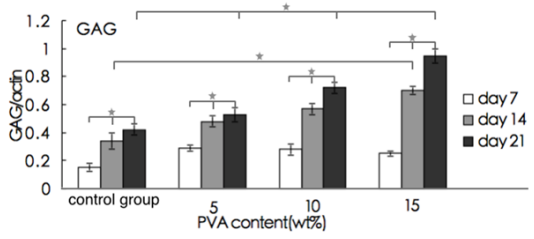

Figure 12. Relative expression GAG of in scaffolds (*P<0.05).

ate. The results of cell culture in vitro showed that the composites not only favored the adhesion and proliferation of seeded human MSCs, but also promote the differentiation toward chondrocytes. In addition, when the mass fraction of PLGA was 30 wt%, and nano-HA 5 wt%, the relatively suitable proportion of PVA was 15 wt% considering morphological properties and short-term practicability in vitro. Overall, all these results suggested that the feasibility of these novel composites as promising scaffolds in cartilage tissue engineering.

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Disclosure of conflict of interest

None.

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