Mineralized collagen scaffold bone graft accelerate the osteogenic process of HASCs in proper concentration

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**ABSTRACT**

**Purpose:** To investigate the feasibility and the optimum condition of human adipose-derived stem cells cultured on the mineralized collagen material; and to further explore the mechanism of osteogenic differentiation of the human adipose-derived stem cells stimulated by the mineralized collagen material.

**Methods:** Primary human adipose-derived stem cells (HADSCs) were isolated from human adipose tissue using centrifugal stratification, which had been passed repeatedly to later generations and purified. Human adipose-derived stem cells were cultured on the bone graft material and the optimum concentration was explored by Alamar blue colorimetric method. The rest experiment was conducted according to the result. The experimental groups are shown below: group A (HADSCs + bone graft material); group B (HADSCs). Morphological observation was taken by scanning electronic microscope (SEM). Alkaline phosphatase activities were tested by histochemical method. Calcium deposition was investigated by alizarin red staining. The quantity access of osteogenic-related mRNA: ALP (alkaline phosphatase), BMP2 (bone morphogenetic protein 2) and RUNX2 (runt-related transcription factor 2) were detected using RT-PCR.

**Results:** The cultured cells grew stably and proliferated rapidly. The optimum condition was 0.5 mg/cm² bone graft material coated on the bottom of medium. After culturing on the material 14 days, the alizarin red staining showed that more calcium deposition was detected in group A and alkaline phosphatase activities of group A was higher than group B (p > 0.05). Similarly, after culturing for 14 days, the ALP, BMP2 and RUNX2 transcription activity of group A was higher than group B (p > 0.05).

**Conclusion:** Human adipose-derived stem cells cultured on bone graft material were dominantly differentiated into osteoblast in vitro. Thus it provided a new choice for bone tissue engineering.

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1. Introduction

In the clinical work, several diseases might cause bone defects, such as trauma, infection, tumor, or others. Luckily, most of bone defects could be reconstructed spontaneously. However, in clinical observation, there are still some critical bone defect cannot heal spontaneously thus leading to nonunion prognosis. Numerous conditions could alter the fate of the bone defect, including the size of defects, biomechanical properties, wound environment, surgical technique, metabolic factors, hormones, nutrition, and applied stress [1,2]. In addition, in view of the advantages of enhanced recovery after surgery (ERAS), the long recovery time is a huge loss both for patients and surgeons. To accelerate the natural bone formation and to fill the void, bone tissue engineering has become a highly active research area. Implantation, as a major part of this multidisciplinary subject, has become the most essential method to promote bone regeneration.

Considering the quality of reconstruction, immunological rejection as well as pathophoresis, autogenous bone is the best option of bone graft. Nevertheless, the complications limit its clinical application. Myeroff, C. et al. [3] defined the complications as minor and major. Minor complications were defined as those that required no or minimal treatment and resulted in no long-term disability, while major complications represented those that

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required repeat surgery, readmission, or a prolonged hospital stay or resulted in long-term disability. The review conclude that minor complications have been reported to occur in 7.1%–39% of patients and major complications, in 1.8%–10% of patients. Therefore, the substitution for autogenous bone is the main direction of bone tissue engineering.

In our clinical study, we have applied the mineralized collagen material (BonGold™ mineralized collagen scaffold bone graft, Allgens Medical, Inc.) in many fields, such as filling irregular bone defects, application in minimally invasive surgical, gluing autogenous bone pieces, loading drugs in infected bone defect and complexing with other artificial bone. This material consists 45% type-I collagen and 55% nano-sized hydroxyapatite. In a retrospective study, Huang, C., et al. [4] reported 89 cases using a mineralized collagen graft during revision total hip arthroplasty (THA) as a filler of massive bone defects. The prognosis was favorable (Mean Harris hip scores were 42.5 ± 3.5 before operation, 75.2 ± 4.0 at 10th month and 95.0 ± 3.6 at the final follow-up) and showed no significant complications for 33.6 ± 2.4 months. Similarly, in recent studies, the inductive osteogenesis potential of mineralized collagen to mesenchymal stem cells (MSCs) was assessed and all of them reported a promising candidate for use as a scaffold in bone tissue engineering [5–7]. However, osteogenic induction capacity is not a prominent feature of the mineralized collagen material. Therefore, we believe that the osteogenic induction ability of the mineralized collagen scaffold bone graft can be enhanced in combination with the new technology and the bone healing process can be further accelerated.

In order to improving the biological properties of bone tissue engineering materials, the combination of stem cells is a hot topic. Human Adipose-derived stem cells (HASCs) have been proven the capacity of osteogenic differentiation [4]. In addition to having the differentiation capacity of adipogenic, osteogenic, myogenic and chondrogenic, as the bone mesenchymal stem cells (BMSCs) have, the HASCs also have the many advantages [5]. Firstly, the HASCs are easy to obtain without additional invasive operations, which means lower pain and incidence of complications. Second, the HASCs have shown higher viability and proliferation rate. Third, the ethical permission for medical purpose is easy to obtain. These characteristics make HASCs a good application prospect in bone tissue engineering.

Therefore, combining with the HASCs, the deficiency of osteogenic induction ability of mineralized collagen scaffold bone graft might be remedied. However, the effect of the material on cell viability and differentiation of HASCs are still unknown. In order to investigate the capacity of inducing bone formation and most suitable concentration of mineralized collagen for HDSCs cultured on the surface of the material, a pilot experiment was processed before our study to determine the material concentration for each group. And as a result, the concentrations of each group we chose are 0, 0.5, 2.5, 12.5 mg/cm² and 12.5 mg/cm², respectively.

### 2. Methods

#### 2.1. Material

Materials were selected according to the pilot experiment. The mineralized collagen scaffold bone graft (Product code: G240520 Type Re-7, BonGold™ mineralized collagen scaffold bone graft, Allgens Medical, Inc.) were selected as experimental material. This artificial bone substitute material was widely used in our clinical application. The mineralized collagen scaffold bone graft was a yellow-white porous cube seems like sponge. The cube was crushed into small particles and dissolved in distilled water forming a suspension. After vortex mixing, the suspension was transferred into the culture plate and stood for 3 h. Then the excess liquid was discarded. The material was laid on the surfaces of the culture slide and observed by inverted microscope and scanning electron microscope (SEM). According to the ratio of material mass to base area, the concentrations were divided into four groups: 0, 0.5, 2.5, 12.5 mg/cm². The bottom area of 24-hole cell culture plate was calculated to work out the proper material mass for each group.

#### 2.2. Cell culture of human adipose derived stem cells (HASCs)

HASCs were isolated by Department of Cell Biology of Capital Medical University from the abdominal subcutaneous adipose tissue, as described below. All procedures were consented by the Ethics Committee of Capital Medical University (serial number: 2011SY08).

A proper donor was selected and all stem cells used in latter experiment were from the same donor. The abdominal subcutaneous adipose tissue was put in a culture dish. After soaking the tissue in phosphate-buffered saline (PBS) with 5% penicillin/streptomycin for 5 min, all the blood vessels were removed carefully. The tissue was cut into 1–1.5 mm diameters pieces and then transferred it into a 50 ml centrifuge tube. Centrifuge process was operated after adding PBS with 5% penicillin/streptomycin. The tissue was then digested by type I collagenase for 180 min with the water bath at 37 °C. Rinse the tissue with PBS for 3–5 times after another centrifuge procession. The cells from tube bottom were collected, counted and seeded at a density of 1.5 × 10³/mm² in the proliferation culture medium. The culture dish was incubated at 37 °C and 5% CO₂ for the entire study. Medium was changed every 3 days. Once the cells area was more than 80%, the morphology was observed under optical microscope to ensure that the cells are ready to passage. The cells were detached by trypsin/D-PBS (0.25%, 0.1 ml/cm²) and suspended in culture medium, followed by washing with D-PBS/EDTA (0.2 ml/cm²) (Sigma-Aldrich, St. Louis, MO, USA). The cell suspension was counted and seeded at the proper density in 24-well culture plate.

#### 2.3. Cell viability

To investigate the relationship about the concentration of mineralized collagen material and the cell viability of HASCs, the cells were seeded on different material concentration. A pilot experiment was processed before our study to determine the material concentration for each group. And as a result, the concentrations of each group we chose are 0, 0.5 mg/cm², 2.5 mg/cm² and 12.5 mg/cm², respectively.

After culturing in proliferation culture medium for 7 days, Alamar blue cell viability test was used to determine the influence of

### Table 1

| Primer | Sequence |
|--------|----------|
| ALP Forward primer | 5'-CACATCCTCTCAAGATCCACA-3' |
| ALP Reverse primer | 5'-CCATACGCACACACCTA-3' |
| BMP2 Forward primer | 5'-CTAGTCCCTGGCATAACA-3' |
| BMP2 Reverse primer | 5'-AGCCGATTCCAGGGCTTT-3' |
| RUNX2 Forward primer | 5'-ATGCAGAGGCCAGACCTT-3' |
| RUNX2 Reverse primer | 5'-TTGTTGACAGTTCAGGAG-3' |
material. The cells were rinsed with warm PBS with 5% penicillin/streptomycin. Then the pre-mixed Alamar Blue reagent (Thermo Fisher Scientific Inc, USA) with complete media was added to cells and incubated for 1–4 h. The absorbance- or fluorescence-based plate reader was used to gain the data. The color change and increased fluorescence can be detected using absorbance (detected at 570 and 600 nm) or fluorescence (using an excitation between 530 and 560 and an emission at 590 nm) The incubation times could be lengthened for greater sensitivity without compromising cell health. Each 100 μL of cells need to add 50 μL of 3% SDS (dilute by PBS) to terminate the reaction. The most suitable concentration of material was used in the following experiments, depended on the result of cell viability analysis.

2.4. Cell induction culture on mineralized collagen scaffold bone graft

After culturing for 7 days, 1 mL osteogenic induction medium (ThermoFisher Scientific Inc., USA) was added into the plate instead of proliferation culture medium. The compositions of medium were 10 μL penicillin-streptomycin solution, 10 μL glutamine, 2 μL ascorbic acid, 10 μL glycerophosphate, 0.1 μL dexamethasone,
100 μL fetal calf serum (FBS) and 875 μL basal medium. The media was changed every 3 days, cell morphology was observed by optical microscope at the same time.

2.5. Alkaline phosphatase (ALP) activity

After inducing for 14 days, the cell slides were transferred into a new well and rinsed by warm PBS 3 times. The modified GOMORI calcium cobalt method was used to display ALP content, as follows. The cell slides were fixed by 95% ethanol for 10 min. The incubated buffer was then added for 4–6 h. After that, the slides were rinsed by distilled water for 3 times. 2% cobaltous nitrate was then added into the well for 3–5 min. Hematoxylin was used to counterstaining for 1 min. Then the slides were dried naturally and sealed. The slides were observed using optical microscope. Then the semi-quantitative analysis of images was carried out using ImageJ software.

2.6. Analysis of calcium deposition

The alizarin red staining was used for qualitative analysis of calcium deposition. After induction culturing for 14 days, the cells were fixed by 4% paraformaldehyde for 15 min and rinsed by warm PBS twice, then dyed by alizarin red (ThermoFisher Scientific Inc., USA) for 3–5 min. Then the slides were rinsed by warm PBS again and dried at room temperature. Neutral balsam was used to seal the slides. And inverted microscope was used to observe the calcium deposition. Then the semi-quantitative analysis of images was carried out using ImageJ software.

2.7. Real-time polymerase reaction

After induction culturing for 14 days, the expression of ALP, Bone Morphogenetic Protein 2 (BMP-2) and Runx-related transcription factor 2 (RUNX-2) were determined by Real-Time polymerase Chain Reaction. The primers were designed using Software Primer 5 (Table 1). The total RNA was isolated following the RNeasy Mini Kit (QIAGEN) manufacturer’s instructions. Then the cDNA was obtained by reverse transcription using Superscript III reverse transcriptase and random hexamer primers (Invitrogen). The RT-PCR procedure was operated on a ThermoFisher Scientific applied Biosystems QuantStudio 5 system (Applied Biosystems) using the SYBR Green PCR Master Mix (Applied Biosystems) subsequently.

2.8. Statistical analysis

Each test was conducted for three times and mean value was used in statistical analysis (n = 3). The statistical analysis was performed with SPSS19.0 software and all data were presented as means ± stand errors (SD). Statistically significant different in different groups in our study were evaluated using the paired Student’s t-test, with statistical significance established at p < 0.05.

3. Result

3.1. Material

The original shape and suspension were recorded (Fig. 1A and B). The components of the material were 45% type-I collagen and 55% nano-sized hydroxyapatite. The suspension was then added into the plate and agitated the plate in order to making the material distributing evenly (Fig. 1C). After the excess liquid was discarded, the material was observed by SEM. The block mass structure was the mineralization part and the collagenous fibers hold them together.

3.2. Cell morphology and identification

The primary isolated cells had major variation between individuals (Fig. 2A). After culturing for 24 h, the cells were shaped as fusiform, polygons and stellate, with many cytoplasm ectopma. 6 days later, the cells grew significantly faster and formed colonies of different sizes that spread out. The cells in colonies were arranged in swirs or in parallel (Fig. 2B). The cells area was more than 80% after
culturing for 2 weeks. The cells were then successfully passaged. And the morphology became more homogeneous which mainly present a spindle shape, with fewer polygons and stellate included.

3.3. Effect of material concentration on cell activity

The concentrations of material were divided into 4 groups: 0.5 mg/cm² (Fig. 3A), 2.5 mg/cm² (Fig. 3B), 2.5 mg/cm² (Fig. 3C) and control group (0), respectively. The cells were then added into the plate. After culturing for 7 days, we find that high concentration of the material impedes observation (Fig. 3D). The cell viability was accessed by Alamar Blue Assays. The result showed that high concentration of material inhibited cell growth. The students T test was used to analyze the difference between each experiment group and control group. At the concentration of 12.5 mg/cm², the cell viability was significantly decreased (p > 0.01) (Fig. 3E). At the concentration of 2.5 mg/cm², the cell viability was still significantly decreased compared to the control group (p > 0.05) (Fig. 3E). And the group of 0.5 mg/cm² concentration show no significant limit to the cell viability (p > 0.05) (Fig. 3E). The group of minimum concentration (0.5 mg/cm²) was selected to carry out the next experiment.

3.4. Cell differentiation

To access the ALP activity of HASCs after culturing for 14 days, the modified GOMORI calcium cobalt method was processed. The ALP was dyed to black and dark black grainy or lamellar precipitate in the cytoplasm. And the amount of precipitate in experimental group was significantly larger than control group (Fig. 4), showed that higher ALP was expressed after culturing on the material. Semi-quantitative analysis showed that the proportion of staining area in experimental group was significantly higher than that in control group (p > 0.05).

To access the calcium deposition of HASCs, alizarin red staining was used 14 days culture in both group. The experiment group had significantly larger calcium depositions than the control group (Fig. 5).
osteoblast precursor cells (MC3T3-E1). And this phenomenon ability and was improved by lots of methods [9]. Hydroxyapatite had a limited osteogenic differentiation inductive properties, morphology, and composition of the material [7]. Our experiment material consisted 45% type-I collagen and 55% nano-sized hydroxyapatite. In previous studies, the type-I collagen had shown osteogenic differentiation induction capacity on BMSCs [7,8]. And as a traditional bone tissue replacement material, the mineralized collagen material (Beijing Allgens Engineering, these advantages mean promising application prospect. The inductive osteogenesis ability of the mineralized collagen material was accelerated with the help of the mineralized collagen material. The inductive osteogenesis ability of the mineralized collagen material is increased significantly by mixing the HASCs and the material. The same result was revealed on genetic level. The RT-PCR results on 14 days showed that the level of osteogenic genes (ALP, RUNX2 and BMP2) were significantly up-regulated. Integrating the views of other articles, it could be the result of the osteogenic differentiation process was accelerated. The expression of ALP and RUNX2 were reported up-regulated on the early stage of osteogenic process [15,16]. The function of BMP2 in osteogenic differentiation process was still unknown, however, comprehensive considering of existing literature, the BMP2 was probably activated on the early stage as well [17,18]. Through this combination, we believe that the osteogenic differentiation process was accelerated by the material at the proper concentration. However, further experiments need to be performed to explore the underlying cell signaling pathways.

5. Conclusion

The purpose of this study was to investigate the feasibility of combining mineralized collagen scaffold bone graft with human adipose-derived stem cell in vitro. The mineralized collagen scaffold bone graft showed limited affection on cell viability at the proper concentration. Osteogenic differentiation procession of HASCs was accelerated with the help of materials in vitro thus providing a new choice for bone tissue engineering. While further exploration was needed to remedy the flaws of our study and certify the mechanism of osteogenic differentiation.

Although our result is promising, the experiment still has limitations. First of all, all the procedures are managed in vitro, which means the result only prove the feasibility of association culturing in theory. In the human body environment, the cells grow in three-dimensional structures. Constructing a three-dimensional structures surface is must for further research. To further investigate the feasibility, constructing a three-dimensional titanium alloy scaffold coating by mineralized collagen material as the substrate of HASCs culturing in vivo and vitro is necessary.

Secondly, we only use two osteoblastic induction methods without traditional inducer: osteogenic induction medium and mineralized collagen material, which leads to a monotony of research methods. It has been proved in theory that combination of multiple method in HASCs osteogenic induction is feasible. Rozila et al. evaluate the osteogenic potential of HASCs co-cultured with human osteoblasts (hOBs) in different HASCs/hOBs ratios on electrospun three-dimensional poly[(R)-3-hydroxybutyric acid] (PHB) blended with bovine-derived hydroxyapatite (BHA) and find that this combination could facilitate osteogenic differentiation activity of HASCs [19]. Kim, T. H. et al. report that graphene oxide (GO) line patterns are effective for modulating the morphology of HASCs. It can enhance the differentiation of HASCs into osteoblasts [20]. Maredzia et al. find that adding a 0.5T static magnetic field (sMF) during the culturing procession of HASCs could increase the viability and proliferation rate [21]. Ehnert, S., et al. prove that the exposure to extremely low frequency pulsed electromagnetic fields could improve osteogenic differentiation of HASCs [22].

Then, the goal of our study is to understand the effect of mineralized collagen material on the differentiation and cell proliferation of HASCs. However, due to the deficiency of experimental verification method, we cannot exclude the possibility of differentiation into other cells. Meanwhile, the measurement of alkaline

3.5. Osteogenesis-related gene expression

After culturing for 14 days, the expression of ALP, RUNX2 and BMP2 was accessed by RT-PCR. The results showed that gene expression increased significantly after 14 days of culture on the material (Fig. 6).
phosphatase activity is a single experiment, which could lead a higher probability of random error. Thus, a multi-method experiment is essential in further research. Cowden, K et al. and Fan et al. [23,24] use scanning electron microscopy (SEM) for investigate the morphology and dimensions of the structure, which could obtain more information than the traditional light microscope such as details of material and more accurate cell morphology.

Finally, our study is basic on experiment in vitro and is still under theoretical. The further investigate of clinical application in bone tissue engineering need well-designed animal experiment.

Declaration of competing interest

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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