Int J Clin Exp Med 2019;12(7):8718-8724
www.ijcem.com /ISSN:1940-5901/IJCEM0071936

Original Article

Effect of simulated microgravity on human chondrocyte-like cells

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Received January 9, 2017; Accepted September 10, 2018; Epub July 15, 2019; Published July 30, 2019

Abstract: Background: Cartilage tissue engineering shows a promising prospect for human cartilage reconstruction. However, the effect of microgravity rotating culture system on the proliferation of tissue-engineered human chondrocyte-like cells remains poorly understood. Materials and methods: Chondrocyte-like cells differentiated from human multipotent adult progenitor cells were seeded in the three-dimensional composite scaffold. They were cultured for three weeks under simulated microgravity environment by rotating cell culture system or under the static environment. Structure of complexes was determined by scanning electron microscopy, while HE-staining, immunohistological staining for type II collagen and MTT assay of both group samples were conducted after three-week culture. Results: Micrographs under scanning electron microscopy represented the structure of scaffold, in which chondrocyte-like cells appeared. HE-staining analysis exhibited more cells within the scaffold in the simulated microgravity group than that in the control group. The immunohistological staining result showed that the contents of collagen type II were higher in chondrocyte-like cells exposed to simulated microgravity. The chondrocyte-like cells proliferated faster in the simulated microgravity group than that in normal group as demonstrated by MTT assay. Conclusions: Simulated microgravity promotes the proliferation and matrix production of tissue-engineered human chondrocyte-like cells, suggesting the promising prospect of simulated microgravity by rotating cell culture system combined with tissue-engineered cartilage for human cartilage reconstruction.

Keywords: Rotating cell culture system, simulated microgravity, cartilage tissue engineering, articular cartilage defect, three-dimensional composite scaffold

Introduction

With the acceleration of social changes and aged tendency of population, articular cartilage injury frequently occurs resulting from excessive activity, trauma or degeneration of chondrocytes. However, articular cartilage is composed of hyaline cartilage without blood vessel, nerves and lymphoidtissue, thus leading to limitation of self-repair potential. Consequently, repair or replacement is generally necessary for the treatment of damaged cartilage. Currently, treatments for damaged articular cartilage include drugs and physical therapy [1, 2], arthroscopic lavage and debridement [3, 4], cartilage graft [5]. Nevertheless, several disadvantages are found in these treatments, such as deficient and inconsistent in long-term repair and especially incompletely resolve the problems such as immune rejection after allogeneic transplantation.

Cartilage tissue engineering using a cell-scaffold approach has emerged as a new multidisciplinary field, showing potential for the effective regeneration and repair of damaged articular cartilage. Cartilage tissue engineering aims at recovering the function of tissue by recombining the chondrocyte and extracellularmatrix. Cartilage tissue engineering develops so rapidly and has been brought into clinical trial or applications in repairing all kinds of cartilage defects of large area, e.g. articular cartilage and thyroidcartilage [6]. Cellular selection, scaffold design and biological stimulation are the key points of tissue engineering, and also bring the challenges [7].

Multipotent adult progenitor cells (MAPCs) is a kind of adult stem cells derived from many types of tissues including bone marrow and brain, which is capable of prolonged culture with differentiation potential being maintained

[8, 9]. It has been reported that MAPCs could be induced to be differentiated into various types of cells such as smooth muscle cells [10] and neurons [11]. Therefore, it may be a promising candidate of seed cell for cartilage tissue engineering.

Three-dimensional osteochondral composite scaffold was generated by a TheriForm™ three-dimensional printing process [12]. Cartilage region of the scaffold was 90% porous and composed of D, L-PLGA/L-PLA, with macroscopic staggered channels to facilitate the seeding of chondrocytes into the center of the cartilage portion and allow the transport of nutrients to the cells as well as removal of cellular and polymer degradation by-products. The bone portion was made up of L-PLGA/TCP composite with the aim to maximize bone in growth and maintain critical mechanical properties. The transition region between these two sections was designed to prevent delamination. This scaffold serves as a fully resorbable biphasic synthetic scaffold with good biocompatibility, the structure of which resembles the normal articular cartilage. The rotating cell culture reactor (RCCS) can simulate the microgravity environment for the chondrocytes *in vitro* and be more effective than routine three-dimensional static culture. RCCS, developed by NASA, is often referred to a rotating wall vessel bioreactor. The vessel for cell culture rotates around the horizontal axis, permitting gas exchange through a permeable hydrophobic membrane. Besides, culture medium is mixed gradually through the rotation, leading to a uniform internal environment, ensuring sufficient material transfer [13-15]. Many studies have also demonstrated that the microgravity environment is effective for the construction of tissue-engineered cartilage *in vitro* [16-19].

In this study, chondrocyte-like cells differentiated from human MAPCs were seeded in the three-dimensional composite scaffold and cultured in simulated microgravity environment by rotating cell culture system. The biological characteristics of chondrocyte-like cells were evaluated and the expression of a specific cartilage extracellular matrix protein (collagen type II) in the different culture groups was determined to investigate the effect of the microgravity rotating culture system on the proliferation of tissue-engineered human chondrocyte-like cells.

Materials and methods

Differentiation and cultivation of chondrocyte-like cells

Chondrocyte-like cells were differentiated from human multipotent adult progenitor cells (MAPCs) *in vitro* which were obtained from the bone marrow of clinical healthy volunteers [20]. This study was performed with the approval of the Second Hospital of Wenzhou Medical University Research Ethics Committee (L2014-05). Informed consents of volunteers before experiments were collected, and all the relevant data in the experiment are anonymous with no violations of individuals' health, safety and privacy. Cells were cultivated in a specific medium, and then subjected to magnetic activated cell sorting, as our previous work described [21]. The third passage of cells in good condition was sorted out, and cultured in DMEM/F12 basal medium (Gibco) containing 10% fetal bovine serum (Gibco), 1 ng/ml of TGF-β1 and 5 ng/ml of FGF-2 (Peprotech) at 37°C with 5% CO_2.

Seeding of three-dimensional scaffold

Three-dimensional D, L-PLGA/L-PLA composite scaffold (3 mm × 3 mm × 4 mm) were obtained from Laser Rapid Prototyping Center in the Tsinghua University. Before use, scaffolds were pre-wetted in 75% ethanol for 30 min for three times, followed by repeated washings with PBS. Afterwards, scaffolds were packed by poly lysine solution to promote cellular attachment, disinfected by ultravioletlight and pretreated in DMEM/F12 medium supplemented with fetal bovine serum (FBS), TGF-β1 and FGF-2 overnight.

Each scaffold was seeded for 6 h with above liquid mixture in incubator at 37°C to facilitate cellular adhesion to the scaffold.

Culture of chondrocyte-like cells

The complexes of scaffold and cells were divided into two groups. In simulated microgravity group, cells were placed in vessels of rotating cell culture system (RCCS, Synthecon Inc., USA) and incubated at 37°C, 5% CO_2, saturated humidity for three weeks. In the normal static culture group, the complexes were placed in 12-well plates and incubated in the above spe-

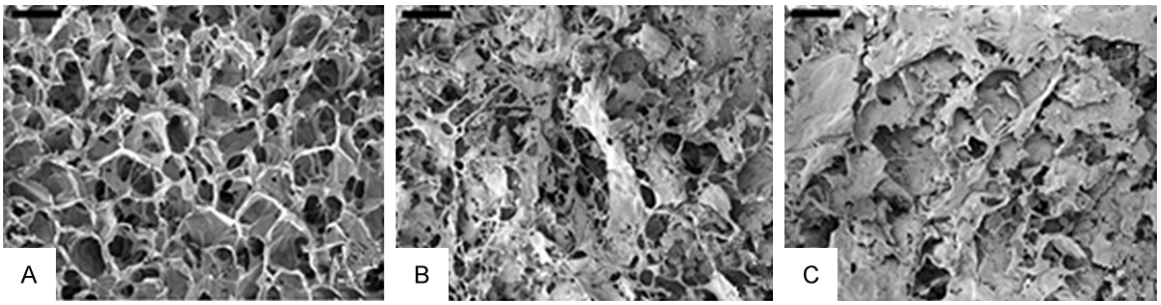

Figure 1. Micrographs under scanning electron microscopy of chondrocyte-like cells/scaffold complexes. A: Scaffold with the big porous and macroscopic staggered channels; B: Chondrocyte-like cells adhered to the scaffold in normal environment; C: Chondrocyte-like cells adhered to the scaffold under simulated microgravity culture. Bar scales: 200 μm.

cific medium for three weeks at 37°C, 5% CO_2, saturated humidity.

The solutions were changed every other day for the first three days, then changed every day thereafter.

Observation under scanning electron microscopy

Micrographs of chondrocyte-like cells which attached to the scaffold under scanning electron microscopy (SEM) were taken following three weeks of culture. Samples were fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in PBS, dehydrated in a graded ethanol series and transferred to isopropyl acetate for substitution. Then, the samples were dried with a critical point dryer (Samdri-790; Tousimis Research Corp, Rockville, MD) and sputter-coated with gold using a Sputter Coater (Desk II, Denton Vacuum, Cherry Hill, NJ, USA). A JEOL 100S (Tokyo, Japan) electron microscope was utilized to image samples.

Histology

The samples were fixed in 4% neutral buffered formalin, dehydrated, and embedded in paraffin. The sections were cut at a thickness of 5 μm, stained with Hematoxylin-eosin solution, and observed under the light microscope.

The expression of type II collagen was analyzed by means of immunohistological staining. The sections were dewaxed in xylene and hydrated in a graded series of alcohol. 0.3% hydrogen peroxide in PBS was used to block endogenous peroxidase activity. The sections were incubated with a mouse polyclonal antibody (Gibco) overnight at 4°C after blocking with goat serum (1:100 dilution). After being rinsed three times in PBS, the sections were incubated with secondary anti-mouse IgG for 1 h at 37°C. The staining was developed in DAB solution, and the sections were counter-stained with hematoxylin and viewed under the light microscope.

MTT assay

An MTT-based assay was employed to estimate cellular activity and spatial distribution. In brief, samples were incubated in MTT solution (0.5 mg/ml in 2% fetal bovine serum culture medium) (Sigma) at 37°C with 5% CO_2 for 2 h and washed in PBS. The insoluble precipitant was extracted in isopropanol for 24 h at room temperature, and the optical density (OD) was determined at a wavelength of 540 nm. Growth curve was drawn with time as abscissa and average optical density as ordinate. Chondrocyte-like cells seeded in three-dimensional D, L-PLGA/L-PLA composite scaffold were cultured statically as a control.

Statistical analysis

Data were analyzed using SPSS software 19.0 (SPSS Inc., Chicago, IL, USA) and presented as means ± standard deviations (SD). Student *t*-test was performed for comparison between the static and RCCS cultured samples. $P < 0.05$ was considered statistically significant.

Results

Chondrocyte-like cells under scanning electron microscopy

Figure 1A depicted the images of three-dimensional composite scaffold under scanning electron microscopy (SEM) with the big porous and

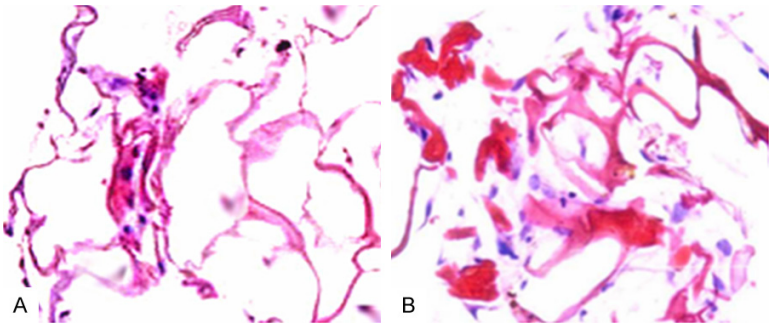

Figure 2. HE staining of chondrocyte-like cells/scaffold complexes after three-week culture. A: In simulated microgravity environment (× 200); B: In normal static environment (× 200).

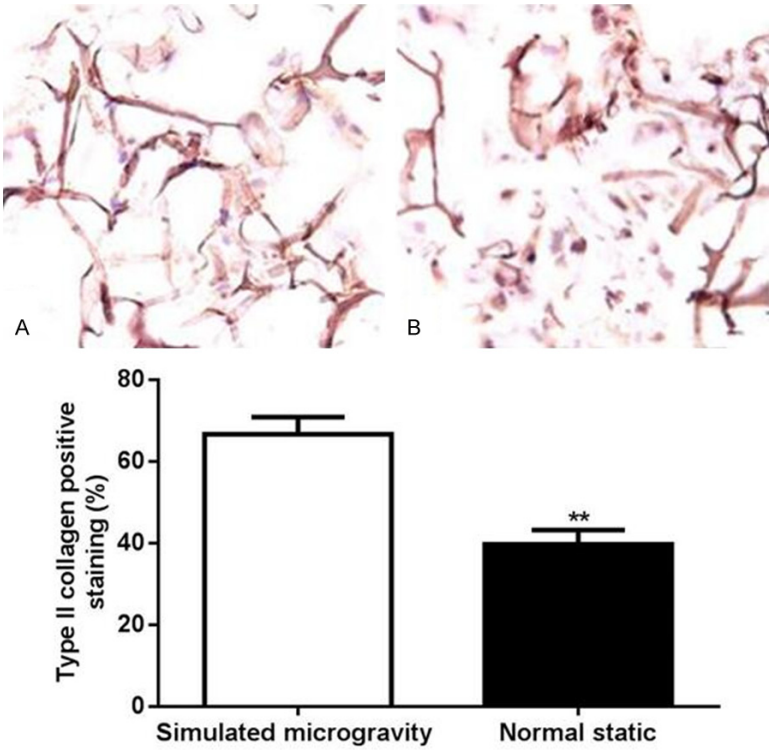

Figure 3. Type II collagen immunohistochemical staining of chondrocyte-like cells/scaffold after three-week culture. A: In simulated microgravity environment (× 200). B: In normal static environment (× 200). Compared with Stimulated microgravity, **P < 0.01.

macroscopic staggered channels. SEM analysis clearly showed the attachment of seeded chondrocyte-like cells to the scaffold after culture of three weeks (**Figure 1B**, **1C**). **Figure 1B** represented part of porous channel of scaffold where the rest channel was taken up by cells. Besides, it seemed difficult to identify the structure of scaffold in **Figure 1C** because of the attachment of seeded chondrocyte-like cells on the scaffold and the massive deposition of extracellular matrix. Our data indicated that the proliferation of chondrocyte-like cells in the simulated microgravity culture was remarkably increased. In addition, equal distribution of cells with more matrix deposition was observed, compared with that in the control group.

HE staining and immunohistochemical staining of collagen type II

In **Figure 2**, large pink area stained by eosin was characterized as the scaffold. Cells grew in the pores, the cytoplasm of which was stained with pink by eosin, while the nucleus and the secreted cartilage matrix were stained with blue by hematoxylin. As we observed, there were much more cells uniformly distributed in the pore of scaffold under simulated microgravity compared with that in normal environment after three weeks of culture (**Figure 2**). In addition, the number of chondrocyte-like cells in simulated microgravity and normal environment can also be reflected by total amount of collagen type II as measured by the immunohistochemical staining (**Figure 3**). Apparently, the contents of collagen type II were significantly higher in chondrocyte-like cells exposed to simulated microgravity ($P < 0.01$), implying simulated microgravity promotes matrix secretion and deposition of surrounding cells.

Evaluation of cell vitality by MTT assay

MTT method was used to estimate the vitality of the chondrocyte-like cells within microgravity after incubation of 1, 3, 6, 9, 12, 15, 18, 21 days. The result indicated that cellular proliferation was remarkably enhanced under simulat-

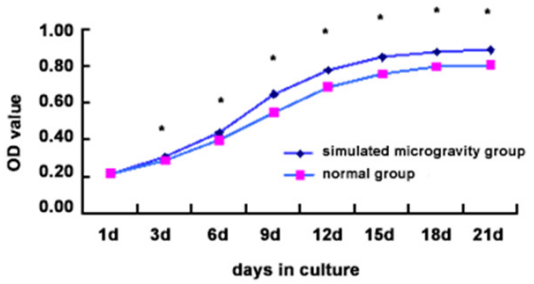

Figure 4. The growth curve of human chondrocyte-like cells seeded in the scaffold in either simulated microgravity environment or normal static environment. *t* test was used for comparison between the static and RCCS cultured samples. *, $P < 0.05$.

ed microgravity (**Figure 4**). There was significant difference ($P < 0.05$) between simulated microgravity groups and control groups except the first day.

Discussion

Based on previous findings that chondrocytes in articular cartilage are inclined to be dedifferentiated into fibroblastic cells with limited chondrogenic potential in two-dimensional cultivation and yet chondrocytes maintain their chondrogenic potential in a three-dimensional environment [22, 23], we used three-dimensional D, L-PLGA/L-PLA composite scaffold according to a previous study [12]. Our data demonstrated that chondrocyte-like cells preferentially seeded into composite scaffold and proliferated in good condition during three-week culture, reflecting favorable biocompatibility between chondrocyte-like cells and composite scaffold. D, L-PLGA degraded into harmless DL-lactic and glycolic acids, while PLA gradually degraded and finally turned into CO_2 and H_2O. Nevertheless, the degradation of composite scaffold was accompanied with the accumulation of these degradation products, which may lead to the gradual decrease in the pH and further impede cellular growth. We did not observe any evident scaffold degradation characteristics during this study.

Rotating cell culture system (RCCS) is often used for simulation of the microgravity environment. In addition, maintenance of a fluid orbit provides low-shear and low-turbulence environment which reduces mechanical damage to the cells and allows more effective transport of material, thus facilitating cellular proliferation and function [15]. In contrast, cells in static three dimension system *in vitro* are unevenly distributed and the growth is relatively slow. Cells outside of the scaffold receive adequate nutrition with more matrix secretion and deposition, thus impeding air exchange and limiting the penetration of nutrients to the center of scaffold to some extent. Furthermore, cellular metabolic waste in the center cannot be promptly discharged, resulting in certain cytotoxicity which hampers the cellular proliferation [24, 25].

It has been demonstrated that microgravity rotating culture system significantly enhanced the chondrogenic effect of TGF-b1 on rabbit bone marrow mesenchymal stem cells *in vitro* as indicated by increased expression of mRNAs and proteins of collagen II and aggrecan [18]. Previous evidence showed that microgravity rotating bioreactor promoted the re-differentiation of rat chondrocytes which were seeded within PLGA sponge, resulting in formation of hyaline-like cartilage *in vitro* [26]. Yu et al. [27] also reported that the dedifferentiated human articular chondrocytes could regain the differentiated phenotype if cultured in the microgravity environment using the rotational cell culture system (RCCS). Based on above findings, we believed that microgravity rotating culture system in our study may prevent the de-differentiation of chondrocyte-like cells and favor the maintenance of their phenotype. Our study elucidated that higher rate of chondrocyte-like cells proliferation was performed under microgravity circumstance with growing amounts of type II collagen in contrast to static environment, which is in agreement with the previous study showing that simulation of microgravity with a rotating bioreactor enhanced proliferation and metabolic activity of chondrocytes stem from bovine and human [28].

Regarding the culture conditions, transforming growth factor-β (TGF-β), as a multifunctional cytokine, plays an essential role in regulating cellular growth and differentiation. It was previously reported that TGF-β1 could promote chondrocyte proliferation, differentiation as well as the synthesis and deposition of the extracellular matrix [29, 30]. In addition, chondrocyte growth was stimulated in the presence of fibroblast growth factor 2 (FGF-2) [31]. However, the exact mechanism by how TGF-β1

and FGF-2 are involved in promoting the growth of human chondrocyte-like cells in vitro culture remains unclear and requires further investigation.

The combination of simulated microgravity by RCCS and scaffold in our study created a three dimensional dynamic condition for human chondrocyte-like cells *in vitro* which promoted proliferation and growth of cells, suggesting their promising prospect for human cartilage repair and reconstruction. Further long-term investigations are required to identify the proliferation of implanted tissue engineered chondrocytes and degradation of corresponding scaffold *in vivo* after simulated microgravity culture.

Acknowledgements

This work was supported by Wenzhou Science and Technology Bureau Foundation (No. Y20140714).

Disclosure of conflict of interest

None.

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