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Original Article

Influence of cell printing on biological characters of chondrocytes

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Abstract: Objective: To establish a two-dimensional biological printing technique of chondrocytes and compare the difference of related biological characters between printed chondrocytes and unprinted cells so as to control the cell transfer process and keep cell viability after printing. Methods: Primary chondrocytes were obtained from human mature and fetal cartilage tissues and then were regularly sub-cultured to harvest cells at passage 2 (P2), which were adjusted to the single cell suspension at a density of 1×10^6/mL. The experiment was divided into 2 groups: experimental group P2 chondrocytes were transferred by rapid prototype biological printer (driving voltage value 50 V, interval in x-axis 300 μm, interval in y-axis 1500 μm). Afterwards Live/Dead viability Kit and flow cytometry were respectively adopted to detect cell viability; CCK-8 Kit was adopted to detect cell proliferation viability; immunocytochemistry, immunofluorescence and RT-PCR was employed to identify related markers of chondrocytes; control group steps were the same as the printing group except that cell suspension received no printing. Results: Fluorescence microscopy and flow cytometry analyses showed that there was no significant difference between experimental group and control group in terms of cell viability. After 7-day in vitro culture, control group exhibited higher O.D values than experimental group from 2nd day to 7th day but there was no distinct difference between these two groups ($P>0.05$). Inverted microscope observation demonstrated that the morphology of these two groups had no significant difference either. Similarly, Immunocytochemistry, immunofluorescence and RT-PCR assays also showed that there was no significant difference in the protein and gene expression of type II collagen and aggrecan between these two groups ($P>0.05$). Conclusion Cell printing has no distinctly negative effect on cell vitality, proliferation and phenotype of chondrocytes. Biological printing technique may provide a novel approach for realizing the oriented, quantificational and regular distribution of chondrocytes in a two-dimensional plane and lay the foundation for the construction of three-dimensional cell printing or even organ printing system.

Keywords: Chondrocytes, cell printing, tissue engineering, proliferation, viability

Introduction

Cartilage defects have always been a challenging problem in surgical treatment. Traditional tissue engineering technology provides a new way for repairing cartilage defects [1, 2], while traditional tissue engineering also have limits, it is restricted in precisely placing different cell types and extracellular matrix at the same time directly into three-dimensional (3D) scaffolds. Therefore engineered production of artificial tissues and organs cannot be realized by traditional tissue engineering method.

However, the emergence of cell printing technology, which is considered to be one of the most promising technologies in tissue engineering, provides a novel approach for the production of artificial tissues and organs. The basic principle of bio-printing technology is to form a three-dimensional multicellular system containing precisely positioned cells, biological factors and biomaterial scaffolds in computer-aided layer-by-layer assembly. Furthermore, simultaneous polymerization during 3D printing is critical to the maintenance of precise positions of deposited cells and biomaterial scaffolds during layer-by-layer assembly. By bio-printing technology human functional microvasculature for thick and complex tissues have been fabricated successfully [3].

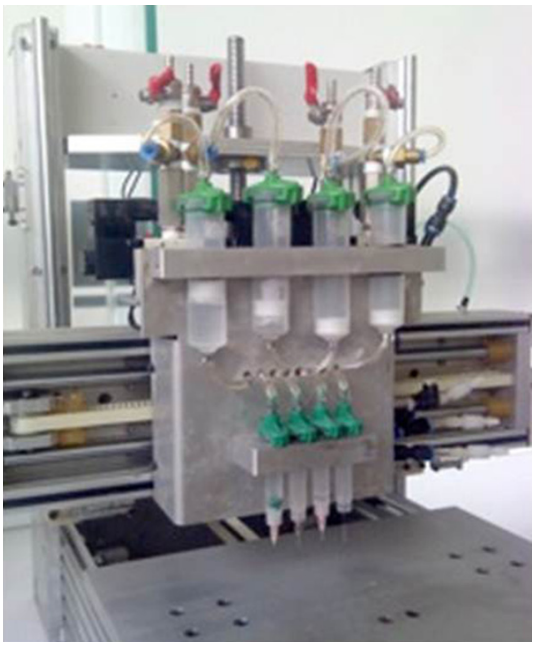

Figure1. 3D cell printer with 4 nozzles for ejecting 4 types of materials/cells.

Among various printing methods, inkjet printing is a widely used technology in cell printing. Inkjet printing is a noncontact printing technique that replicates digital pattern information onto a substrate with tiny ink drops [4]. Air bubbles generated by heating in the printhead collapse to provide pressure pulses to eject ink drops with various volumes ranging from 10 to 150 pL [5, 6]. Although the heating factor in each nozzle raises the local temperature to 300°C and lasts for a few microseconds during printing process [7], ejected cells are heated for only 2 μs with a temperature rise of 4°C-10°C above ambient and an average cell viability of 90% [8]. But the heat generated by printing, the voltage that drives printing and the stress force generated during cell fall from nozzle to the substrate, all of these factors will exert negative effects on cell viability and characterization. How to avoid cell damages during printing process and maintain cell phenotype is one of the key problems that need urgently to be solved for engineered bio-printing technology.

The objective of this study is to test the feasibility of thermal inject-based bio-printing technology in the engineering of cartilage tissues. The criteria for success are to preserve cell viability and maintain chondrogenic phenotype by adjusting appropriate parameters of inkjet printer, all of which are met.

Methods

Cell harvest and culture

For human cartilages, mature tissues derived from excised microtia cartilage were donated by patients who underwent plastic surgery of ear reconstruction with informed consent, and fetal tissues from aborted fetuses (about 16-20 weeks) were donated by patients with informed consent for research purpose only. Human tissue handling was approved by the Ethics Committee of Shanghai 9th People's Hospital.

As described previously [9], obtained cartilage tissues were cut into 2×2 mm^2 pieces and washed with phosphate buffered saline (PBS). Afterwards the cartilage pieces were digested with 0.25% trypsin plus 0.02% EDTA in PBS at 37°C for 30 min and then were further digested with 0.1% collagenase II (Serva, Germany) in serum-free Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) at 37°C on a shaker for 12~16 h. After centrifugation, chondrocytes were collected, resuspended and cultured at the density of $2.0\times10^4/cm^2$ in DMEM containing 10% fetal bovine serum (FBS, Gibco, USA) and antibiotics (penicillin 100 U/ml, streptomycin 100 U/ml, Gibco, Gland Island, NY, USA) in 10 cm dish at 37°C with 5% CO_2 in an incubator (Forma Scientific, USA). The media were changed every 2-3 days. When confluence reached 80%~90%, cells were subcultured at a ratio 1:3 and passage 2 (P2) chondrocytes were collected.

Cell printing

The rapid prototyping printer (**Figure 1**) was moderately modified for printing cells (60 μm in diameter). To create uncontaminated environment, the ink was emptied and the cartridge was washed thoroughly with 95% ethanol and sterile water for three times. This procedure was previously proven to be enough to create sterile conditions for cell cultures. For cell preparation, passage 2 chondrocytes were obtained and resuspended with high glucose-DMEM (H-DMEM containing 10% FBS) then were adjusted to the single cell suspension at a density of 1×10^6/mL. Afterwards P2 chondrocytes were transferred by rapid prototype biological printer. The printing parameters were set as follows: interval in x-axis 300 μm, interval in y-axis

Table 1. Primers used in RT-PCR and quantitative PCR analysis

Gene	Primer sequence (5'-3')	Product size (bp)	Annealing temperature (°C)
Collagen II	Sense: TCCCCGGCACTCCTGGCACTGAT	510	58
	Antisense: CTTGGGCACCTCGGGCTCCTTTAG		
Aggrecan	Sense: ATGCCCAAGACTACCAGTGG	500	58
	Antisense: TCCTGGAAGCTCTTCTCAGT		
β-actin	Sense: ATCATGTTTGAGACCTTCAA	318	58
	Antisense: CATCTCTTGCTCGAAGTCCA		

1 500 μm, printing pulse 10 μs, frequency 50 Hz, velocity 20 mm/s, driving voltage value 50 V. After printing, cells were collected with 50 ml centrifuge tubes and then centrifuged at 1500 r/min for 5 minutes. After centrifuging the supernatant was removed, cells were resuspened with H-DMEM medium as experimental group. Meanwhile, control group performed the same steps as the experimental group except that cell suspension received no printing.

Cell proliferation assay

100 μl of cell suspension (1500 cells/well) was dispensed in a 96-well plate and the plate was incubated for an appropriate length of time from 1day to 7 days respectively in a humidified incubator (at 37°C, 5% CO_2). Before testing, 10 μl of CCK-8 (Cell Counting Kit-8, Dojindo, Japan) solution was added to each well of the plate. Then the plate was incubated for 2-4 hours in the incubator. A microplate reader (Thermo Electron Corporation, Finland) was used to measure the absorbance at 450 nm.

Fluorescence microscopy by LIVE/DEAD® Viability/cytotoxicity kit

Prepared cells were cultured on sterile glass coverslips inside 6-well plate for 2-3 days until 80%-90% confluence was reached. Then the cells were washed prior to the assay to remove serum esterase activity generally present in serum-supplemented growth media gently with 500-1,000 volumes of sterile PBS. 100-150 μL of the combined LIVE/DEAD® assay reagents (Invitrogen, Carlsbad, CA) was added to the surface of a 22 mm square coverslip, so that all cells were covered with solution. Afterwards the cells were incubated for 30-45 minutes at room temperature. Following incubation, 10 μL of PBS solution was added to a clean microscope slide. Fine-tipped forceps was used to carefully invert and the wet coverslip was mounted on the microscope slide. To prevent evaporation, the coverslip was sealed to the glass slide. A fluorescence microscope was used to view the labeled cells.

Flow cytometry

LIVE/DEAD® assay reagents were also used to perform flow cytometry assay. Calcein AM was diluted in DMSO to make a 50 μM working solution. 1 mL of cell suspension with 0.1 to 5×10^6 cells/mL was prepared for each assay. 2 μL of 50 μM Calcein AM working solution and 4 μL of the 2 mM Ethidium homodimer-1 stock was added to each milliliter of cells and then the sample was mixed. Afterwards the cells were incubated for 15-20 minutes at room temperature, protected from light. Following incubation, stained cells were analyzed by flow cytometry using 488 nm excitation and measuring green fluorescence emission for Calcein (i.e., 530/30 bandpass) and red fluorescence emission for Ethidium homodimer-1 (i.e., 610/20 bandpass). Single color cells were performed as standard compensation. Cell population would separate into two groups: live cells would show green fluorescence and dead cells would show red fluorescence.

RT-PCR

Total RNAs were extracted from the passage 2 (P2) chondrocytes and were reverse-transcribed to cDNA using a RT kit (Takara, Japan) according to manufacturer's protocol.

RT-PCR was performed with a PCR Kit (Takara, Japan) and the amplification was performed by 29 cycles consisting of denaturation at 95°C for 30 s, primer annealing at temperatures listed in **Table 1** for 30 s, primer extension at 72°C for 45 s and termination at 72°C for 10 min. 10 mL of each product were analyzed electrophorestically with a 1% agarose gel stained in ethidium bromide and the band density was analyzed using the analysis software (Tanon, China).

Immunocytochemical (ICC) and immunofluorescent (IF) staining

Chondrocytes of passages 2 were tested for type II collagen (Col II) expression with immuno-

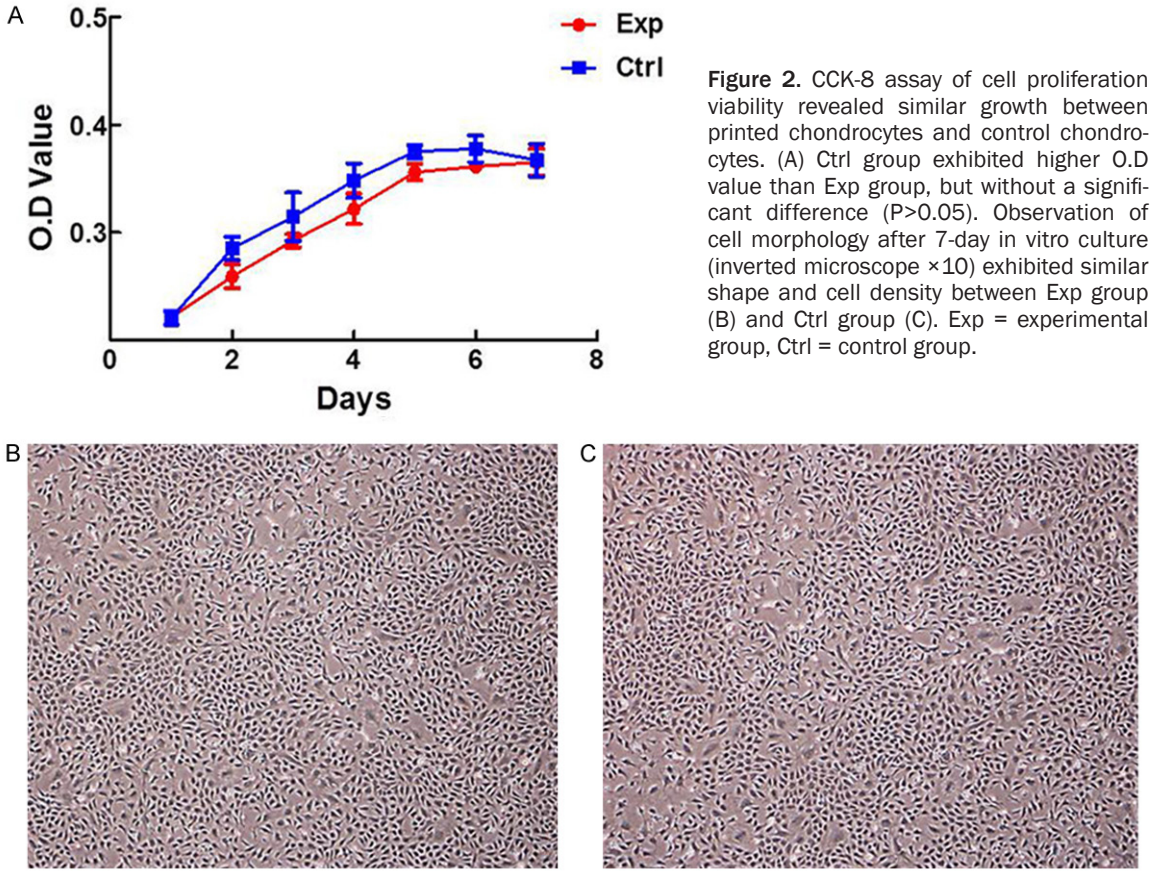

Figure 2. CCK-8 assay of cell proliferation viability revealed similar growth between printed chondrocytes and control chondrocytes. (A) Ctrl group exhibited higher O.D value than Exp group, but without a significant difference (P>0.05). Observation of cell morphology after 7-day in vitro culture (inverted microscope ×10) exhibited similar shape and cell density between Exp group (B) and Ctrl group (C). Exp = experimental group, Ctrl = control group.

cytochemistry. P2 Chondrocytes were grown on cover slides for 24 h, fixed with 4% paraformaldehyde (PFA) in PBS and then incubated with mouse anti-human Col II polyclonal antibody (NeoMarkers Inc, Fremont, CA, 1:100 in PBS containing 0.1% bovine serum albumin, BSA) at 4°C overnight, then followed by horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:200 in PBS) and the color was developed with diaminobenzidine.

For immunofluorescent staining against Col II and aggrecan, chondrocytes in both experimental and control groups were cultured on cover slides for 24 h, fixed with 4% PFA in PBS and then incubated with mouse anti-human Col II polyclonal antibody (NeoMarkers Inc, Fremont, CA, 1:100 in PBS), goat anti-human aggrecan polyclonal antibody (Santa Cruz, CA, 1:100 in PBS) at 4°C overnight. After three washes with PBS, chondrocytes were incubated with FITC-conjugated goat anti-mouse IgG or FITC-conjugated rabbit anti-goat IgG (Santa Cruz, CA, 1:200 in PBS) respectively at 37°C for 30 min and the nuclei were counterstained with PI (Sigma, St. Louis, MO, 1:500 in PBS).

Statistical analysis

Student's t-test was applied to compare the difference of O.D values in cell proliferation assay, cell viability analyses and Col II and aggrecan expression levels between experimental group and control group. The *P*-value less than 0.05 was considered statistically different.

For cell viability, IHC and IF assays respectively, semiquantitative analyses were performed with 5 randomly selected views per slide at appropriate magnification to calculate the positive and negative cell numbers within a photographic area. Dead percentage = number of dead cells/(dead cell number + live cell number). Positive percentage = number of positive cells/(positive cell number + negative cell number).

Results

Influence of cell printing on proliferation and morphology of chondrocytes

The CCK-8 Kit was used to detect O.D values of experimental group and control group from 1-7

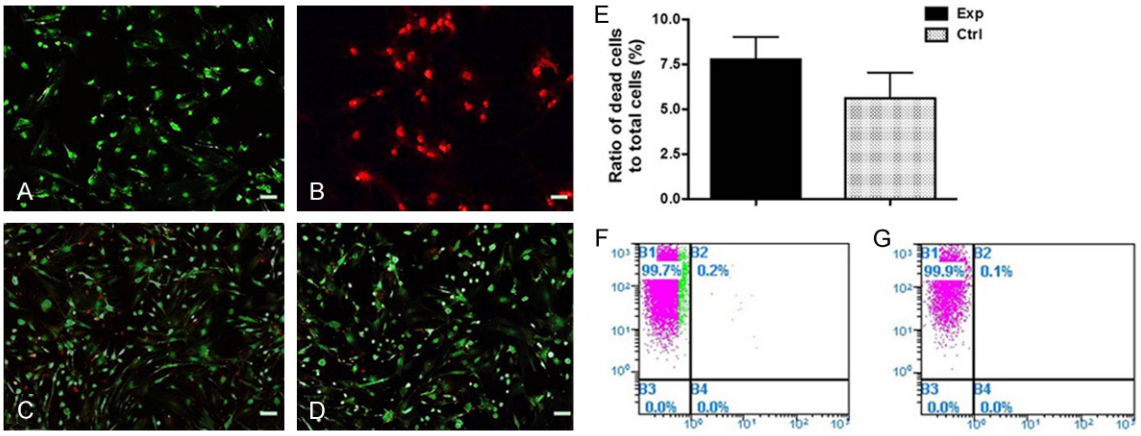

Figure 3. Cell viability test after printing saw no distinctly negative effect on chondrocytes (Laser scanning confocal microscope ×40). Green fluorescence marked cells with normal viability (A), red fluorescence marked dead cells (B), there were a few dead cells in Exp group (C), similar to Ctrl group (D) and semi-quantitative analysis revealed there was no distinct difference between these two groups (P>0.05). (E) Flow cytometry also showed the percentage of live cells in Exp group (F) was as high as in Ctrl group (G). Exp = experimental group, Ctrl = control group.

d. As shown in **Figure 2A**, O.D values of these two groups were about the same on 1st day, while from 2nd day to 7th day, the O.D value of control group was slightly higher than that of printing group, but there was no significant difference between them (P>0.05). This suggested that chondrocytes exhibited similar growth trend as control group after printing, and biological printing did not exert significantly negative effect on cell proliferation.

In addition, inverted phase contrast microscope observation showed that printed chondrocytes could adhere as normal and grew well in elliptical shape after 7-day *in vitro* culture. There was no distinct difference in morphology and cell density between experimental group (**Figure 2B**) and control group (**Figure 2C**).

Viability assay of printed chondrocytes

As immunofluorescence results showed that, live cells labeled green fluorescence (**Figure 3A**) and dead cells labeled red fluorescence (**Figure 3B**). There were a few dead cells among total cells in experimental group (**Figure 3C**), similar to that in control group (**Figure 3D**). Similarly, semi-quantitative analysis also revealed that the ratio of dead cells to total cells in experimental group was slightly higher than in control group, but there was no significant difference between them (P>0.05) (**Figure 3E**).

As shown in **Figure 2**, flow cytometry assay showed similar results. The percentage of live cells in experimental group reached 99.7% (**Figure 3F**), as high as 99.9% in control group (**Figure 3G**).

Characterization of printed chondrocytes

As shown in **Figure 4A**, immunocytochemistry analysis suggested there were still Col II-positive chondrocytes in experimental group. Semi-quantitative assay of ICC showed that the positive percentage reached about 30%, slightly lower than that of control group, but there was no distinct difference in the percentage of positive cells between these two groups (P>0.05) (**Figure 4B**). Moreover, immunofluorescence test also revealed the protein expression levels of Col II and aggrecan in printed chondrocytes (**Figure 5A**) without significant difference between these two groups (P>0.05) (**Figure 5B**, **5C**). In addition, RT-PCR results suggested that printed chondrocytes exhibited similar gene expression levels of Col II and aggrecan as control group (**Figure 6**). As mentioned above, there was no significant difference in the protein and mRNA expression of type II collagen and aggrecan between experimental group and control group.

Discussion

Tissue engineering has emerged as a most promising approach in regenerative medicine.

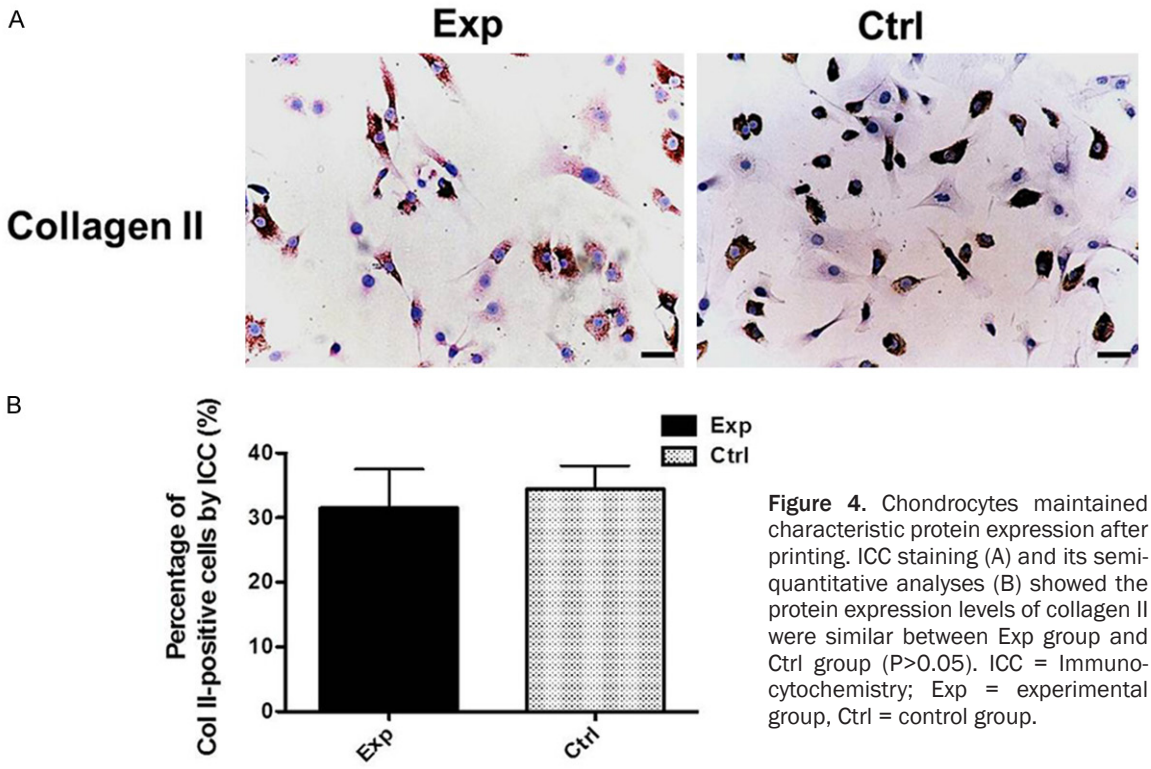

Figure 4. Chondrocytes maintained characteristic protein expression after printing. ICC staining (A) and its semi-quantitative analyses (B) showed the protein expression levels of collagen II were similar between Exp group and Ctrl group ($P>0.05$). ICC = Immunocytochemistry; Exp = experimental group, Ctrl = control group.

The basic principle of tissue engineering, is to adhere tissue cells or stem cells onto the biocompatible biomaterials, to form cell-biomaterial compounds, then transplant the compounds into *in vivo* or *in vitro* microenvironment, then transplanted cells reproduce extracellular matrix while biomaterials gradually degrade at the same time, finally form new targeted tissues with specific morphology or function, aiming to repair or regenerate damaged tissues or organs [10]. But some limits cannot be neglected in traditional tissue engineering technology: (1) it takes a few weeks for engineered tissues or organs to complete differentiation in morphology, mechanics, biochemistry and function; (2) human organs and tissues, made up of a variety of cell types and extracellular matrix, are so complicatedly constructed that it is difficult to perform simultaneous placement and precise position of different factors in a 3D scaffold [11]; (3) lack of blood, oxygen and nutrient supply inside the tissue structures can lead to tissue necrosis [12]; (4) cells permeate to scaffold materials very slowly restricted by the spatial resolution of scaffolds [13]. So it is hard to realize engineered production of artificial tissues and organs.

Currently cell printing has become one of the most potential technologies in biological manufacturing area, which is widely applied in repairing of stent grafts, regenerative medicine and manufacturing of 3D organ printers, etc. As an emerging technology in tissue engineering bioprinting technology is still in the initial research stage though, it has obtained some achievements. It can be used to print different types of cells, which can merge under specific culture conditions to form a complex 3D tissue in the end. J. A. Barron et al. have successfully printed human multilayer osteosarcoma cells on the matrigel and formed a 3D cell structure, and then cell survival test showed that cell survival rates reached as high as 95% after printing [14]. Tao Xu et al. have successfully printed major motor neurons, neurons of cerebral cortex and hippocampus neurons of mouse embryos respectively [15], and later experiment verified printed cells could form a simple structure with certain forms [16]. Ringeisen et al. then succeeded in printing normal cells such as fibroblasts and these printed cells could form some specific 3D structures [17].

However, bio-printing system also encountered some unavoidable defects: (1) the nozzle is

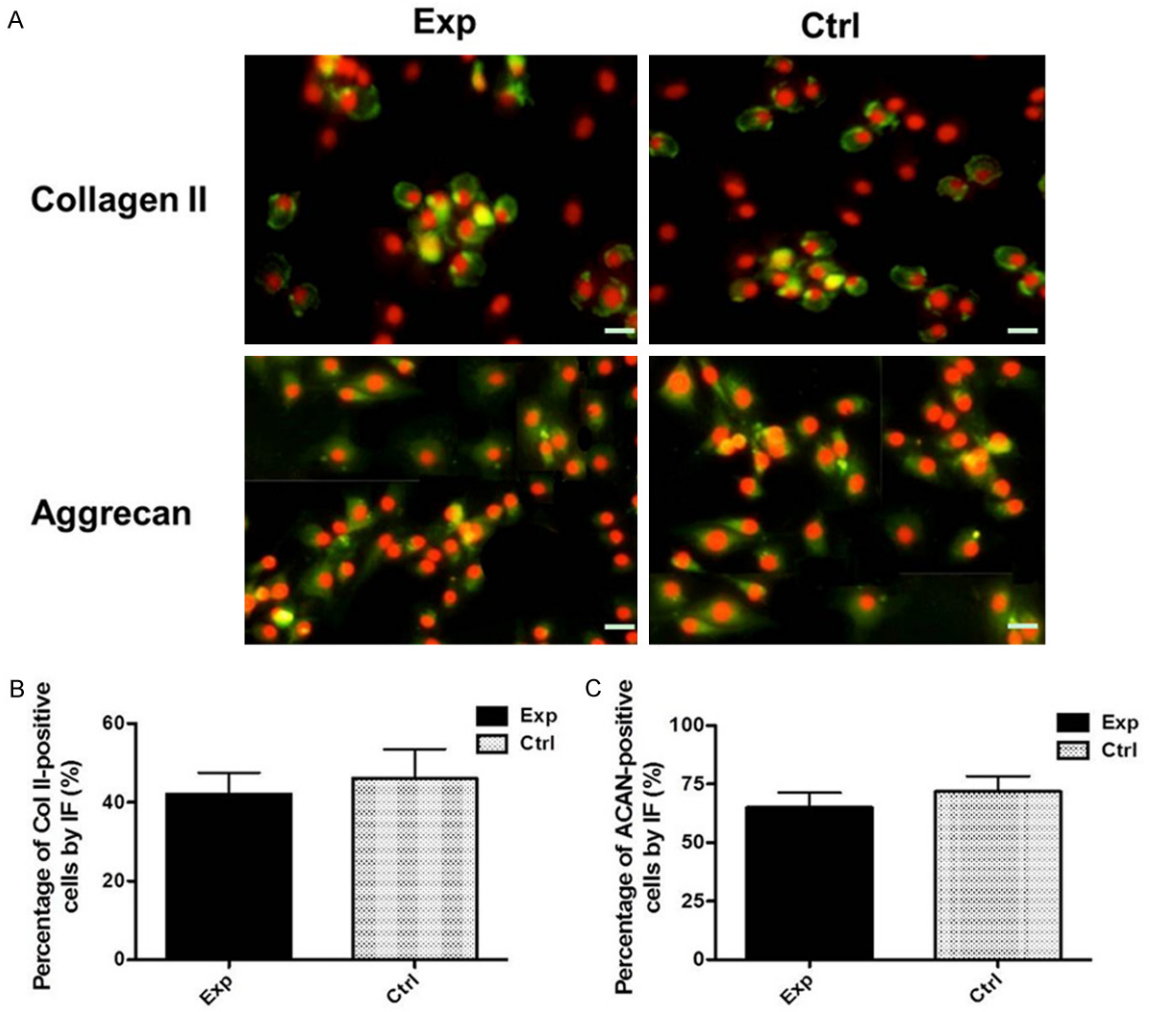

Figure 5. Chondrocytes maintained characteristic protein expression after printing. IF microscopy (A) and their semi-quantitative assays (B, C) also suggested the similar protein expression levels of collagenIIand aggrecan (P>0.05). IF = Immunofluorescence; ColII = typeIIcollagen; ACAN = aggrecan; Exp = experimental group, Ctrl = control group.

easily clogged; (2) the frequent backfill of ink causes the pollution of biomaterials; (3) high viscosity liquid cannot be printed; (4) cell viability declines after printing. In addition, extracellular matrix is necessary for cell printing to achieve long-term culture. One of the most challenging problems is how to reduce cell damages during printing process. Cell survival rate is closely correlated with cell types, features and thickness of coating materials and the changes of external environment. In the process of droplet formation and injection, cells will be affected by shearing stress, mechanical and thermal damages. For instance, in the printing process acceleration velocity of droplets can reach as high as 10^9 ×g, cell viability will be significantly affected [18]. Besides, in cell landing process, thickness of the coating material and jet velocity are two typical influential elements too.

However, so far most cell printing experiments have been designed to test cell viability, while printing methods lack in-depth studies. The influence of printing control parameters such as time interval and driving pulse on cell damages, and single cell suspension density etc. are lack of detailed and theoretical analyses, all of which not only limit its application, but also seriously hinder the development of specialized cell injection device, failing to realize engineered cell printing. Thus, how to realize rapid and accurate position of target cells in target area, and meanwhile maintain cell vitali-

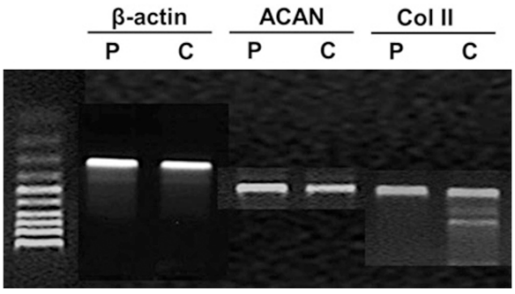

Figure 6. Semi-quantitative analysis of gene expression of chondrocytes after printing. Gene expression of ACAN and Collagen II was observed in RT-PCR examination. Col II = type II collagen; ACAN = aggrecan; P = experimental printing group, C = control group.

ty, cellular morphology and phenotype and continue to grow after printing, by controlling jet process of cells or extracellular matrix during bio-printing, are two key problems.

Since the basic principle of tissue engineering is to regenerate tissues without causing morbidity in donor site [1], it is very likely to be fulfilled in cartilage tissue engineering via this method. For example, human ear-shaped cartilage engineering has been proved for its great potential in clinical therapy of microtia deformity [19], yet, the requirements of significantly high amounts of autologous chondrocytes made it difficult in practical application. Chondrocytes are fully differentiated mature cells, which are characterized by capability to reproduce cartilaginous matrix such as collagen type II and glycosaminoglycans [20] and are often used as seed cells for cartilage tissue engineering either *in vivo* [19] or *in vitro* [21]. Collagen type II [22] and aggrecan [23] are two major cartilage specific matrix molecules. As our study showed that, printed chondrocytes exhibited the protein and mRNA expression level similar to control unprinted chondrocytes (**Figures 4-6**), suggesting printed cells maintained chondrogenic phenotype. Besides, cell viability test (**Figure 3**) and *in vitro* culture results (**Figure 2**) revealed that shortly after printing chondrocytes still kept high survival rates, and furthermore after 7-day *in vitro* culture printed cells also exhibited similar proliferation and cellular morphology as control group.

In our study, printing intervals (including intervals in x, y axis) were determined through two-dimensional designing and adjusting printing parameters, to achieve two-dimensional distribution of "cell ink droplets" in the tissue. Based on the suspension concentrations of printed cells, the number of the "cell ink droplets" was modified, to achieve quantitative and fixed-point distribution in a three-dimensional structure, thus realizing rapid and precise positioning of printed cells in target area. After printing cell viability analysis revealed that cell damages were reduced to minimum standard.

In conclusion, our work demonstrates the feasibility of bio-printing of chondrocytes. By adjusting these bio-printing parameters as well as the components of the bio-ink, we believe that it is possible to construct some kind of complex 3D structures required to heal a wide variety of cartilaginous lesions. Bio-printing based on thermal inkjet printing technology can be a very potential approach for anatomic cartilage engineering and provides necessary capabilities for further 3D printing system.

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Disclosure of conflict of interest

None.

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