Optimization studies on growth of human chondrocytes

Lucky Srivastava1, Ishan S Chandel2, Amit K Rai3, Amit Rastogi4 and Pradeep Srivastava2*

1Surgery Department, Indian Veterinary Research Institute, Izatnagar 243 122, India
2School of Biochemical Engineering, Indian Institute of Technology, Banaras Hindu University, Varanasi 221 005, India
3Centre for Genetic Disorders, Department of Zoology, Banaras Hindu University, Varanasi 221 005, India
4Department of Orthopedics, Institute of Medical Sciences, Banaras Hindu University, Varanasi 221 005, India

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Cartilage is one of the expectative targets for tissue engineering because it differs from other tissues in its limited capacity for self-repair, which makes the treatment of chondral lesions difficult. The techniques currently available to treat cartilage lesions may relieve symptoms, but do not regenerate the injured tissue. Autologous chondrocyte transplantation uses cell biology and cell culture techniques that aim at regenerating the hyaline cartilage and restoring the function of the articular surface. Present study was carried out to standardize and analyze the culture conditions of chondrocytes from human donors and ex vivo expansion of the chondrocytes in view of their therapeutic use. Human chondrocytes, procured and isolated from cartilage piece collected from the patient suffering from cartilage damage, were cultured and subjected to growth studies. Cell culture assays were performed to evaluate isolation, viability, morphology and proliferation. In the present study, the cell isolation process and culture conditions were proved to be efficient and assured a high cell proliferation rate as well as a cell viability of 75-87.5%. The results enabled the evaluation of biopsy processing conditions to obtain chondrocytes, culture conditions for cell proliferation and evaluation of suitable strategies for chondrocyte culture.

Keywords: Cell culture, specific cell growth rate, chondrocytes/transplantation; autologous/methods, viability

Introduction

Articular cartilage is the thin load bearing connective tissue covering the ends of long bones in all diarthroidal joints providing predominantly a mechanical function1. It is a metabolically active tissue that under normal conditions is maintained in a relatively slow state of turnover. Chondrocytes are the sole cell type found within cartilage and control its formation and composition. In humans, chondrocytes represent only about 1% of the volume of hyaline cartilage, but are essential since these cells replace degraded matrix molecules to maintain the correct size and mechanical properties of the tissue. Despite the activity of these cells, even minor lesions or injuries may lead to progressive damage and, in case of articular cartilage, may leads to subsequent joint degeneration because of limited capacity for intrinsic repair or low productivity of matrices in regenerated chondrocytes1,4. The difficulty in the self-repair of cartilage seems to be due to the lack of sufficient supply of healthy chondrocytes to the defective sites4. Cartilage tissue engineering holds the promise to overcome such limitations by employing ex vivo culture techniques and supportive artificial materials6. In principle, when placed in the proper 3D environment, chondrocytes retain their activities.

Damage to cartilage, if left untreated, can lead to irreversible degeneration of the surrounding tissue, what is generally characterized as osteoarthritis (OA). Current treatment options for cartilage injuries, which include joint cleavage, tissue debridement7, micro-fracture of the subchondral bone8, abrasion arthroplasty9,10 and the transplantation of autologous or allogeneic osteochondral grafts, have yielded successful clinical outcomes11. The techniques currently available to treat cartilage lesions may relieve symptoms, but do not regenerate the injured tissue. Autologous transplantation of chondrocytes procedures are cell-based repairs that aims at regenerating the hyaline cartilage and restoring the function of the articular surface12. This advanced technology allows cartilage cells (chondrocytes) to be harvested from knee, cultured and multiplied. The fresh chondrocytes are then re-implanted in knee that cause hyaline-like cartilage to repair the defect in articulating surface13,14. The autologous chondrocyte
implant (ACI) restores the articular surface with one’s own functional repair tissue without compromising the integrity of healthy surrounding cartilage or the subchondral bone.

In the present study, an attempt has been made to improve the cell culture technique aiming at autologous transplantation of chondrocytes and evaluate the cell isolation, proliferation and morphology.

Materials and Methods

Biopsy Collection

Piece of human articular cartilage was collected from the patients suffering from cartilage damage in the Department of Orthopedic Surgery, Institute of Medical Sciences (IMS), Banaras Hindu University, Varanasi. The cartilage samples were collected in culture medium. Thermal containers were used to transport cartilage samples so that the temperature was kept between 4-8°C.

Chondrocyte Isolation

Samples of articular cartilage were taken to the culture laboratory and submitted to enzyme digestion process to isolate cells. Cartilage was minced into small pieces (0.5 mm<sup>3</sup>), washed with DMEM/F12 (Dulbecco’s modified eagle’s medium: Ham’s F12; Sigma) and digested with 0.4% pronase (Sigma) for 1 h, followed by 0.016% collagenase (Sigma) treatment for 4 h; the enzymes were dissolved in DMEM/F12. After digestion, the suspension containing isolated chondrocytes was centrifuged at 1500 rpm for 3 min. The resulting cell pellet was washed twice with DMEM/F12 media to remove remaining digestive enzyme.

Chondrocyte Culture

The chondrocyte cells were cultured in culture flasks at a concentration of 1000 cells/cm² using DMEM/F12 supplemented with 10% FBS (Fetal bovine serum; Sigma), 50 mg/mL of ascorbic acid (HiMedia), 100 mg/mL of antibiotic gentamycin (HiMedia), 0.25 mg/mL of antifungal agent amphotericin B (HiMedia) and 1% MEM vitamin (minimum essential medium vitamin solution; HiMedia), and maintained at 37°C in a humidified incubator at 5% CO2 atmosphere (Thermo Electron Corporation) for 28 d. Cell culture medium was replaced every 3 d.

Cell Count and Viability

Total cells and percentage of viable cells were counted, right after cell isolation and at the end of cultures, using trypan blue vital dye (HiMedia) in a hemocytometer (Paul Marienfeld GmbH & Co.).

The cell generation time (t<sub>g</sub>) was calculated as: 
\[
t_g = \frac{0.693}{\mu},
\]
where \(\mu\) is specific growth rate.

Histological Analysis

Cells were stained with hematoxylin and eosin for observing morphology. Cells were first washed with distilled water, then stained with haematoxylin (HiMedia) for 1-3 min and again rinsed with water. These cells were then placed in acid alcohol (E Merck) for differentiation for 30 sec and rinsed with water. Then counter staining with eosin (HiMedia) was done for 2 min, followed by dehydration and mounting. Stained cells were observed under inverted microscope (Leitz Labovert FS) at 40× magnifications.

Morphological Analysis

The morphological analysis of cells was routinely performed using phase microscopy in an inverted microscope at 40× magnifications. Change in the shape of chondrocytes was routinely observed. The cultures were photographed regularly to record cell morphology.

MTT Assay

The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole] measurements were performed by replacing the DMEM culture medium with MTT solution (E Merck). MTT solution was added to each tissue culture well and mixed by tapping gently on the side of the tray. Then it was incubated at 37°C for 4 h. DMSO was added into each well to dissolve the formazan and extinction was measured at 490 nm.

Results and Discussion

Chondrocytes Isolation

Cells obtained after enzymatic digestions presented rounded morphology, which is the characteristic of these cells. Fig. 1 shows the image of isolated cells viewed under inverted microscope at 40× magnifications. Chondrocyte cultures were maintained for 14 to 40 d. The growing cells were quantified using hemocytometer and trypan blue dye to analyze the cell number per mL as well as their viability. Isolated cells were cultured using growth medium and were quantified on 1<sup>st</sup>, 2<sup>nd</sup>, 7<sup>th</sup>, 14<sup>th</sup>, 21<sup>st</sup>, 28<sup>th</sup>, and 35<sup>th</sup> d of the cultures. Seven batches of isolated chondrocytes were taken, washed and processed for further growth study, and results are shown in Table 1.
The viability of the cells was also calculated on the basis of initial and final number of cells. It was observed that the viability of chondrocytes varied with reference to the period of incubation and growth (Fig. 2). The cell viability was sufficiently good till 24th d, but it started decreasing in the 4th wk (week) of incubation, which might be due to metabolic processes occurring in the cells. The average cell viability, as calculated after 28th d (7 batches), was found to be 82%, which is sufficiently good for chondrocyte transplantation.

**Growth Kinetics**

The growth kinetics of cultured chondrocytes was evaluated in shake flasks. Studies were performed on 5 samples and cell quantification was made with reference to incubation time. The results are presented in Fig. 2, which show that the number of cells increased up to 28 d after which slight decrease was noticed. Overall, the achieved cell viability was satisfactory (>80%). The growth profile of the chondrocytes exhibited Monod’s growth model. It was observed that, with an initial lag phase of growth up to 7 d, the cell multiplication increased with time and a rapid increase was observed in the number of cells up to 28 d, exhibiting the exponential phase of growth. In the exponential phase, the cells utilized available substrate in the medium and the cell number multiplied rapidly. After 28, the cell number declined, exhibiting a stationary phase or decline phase for the chondrocytes.

The MTT assay shows the metabolism of the tetrazolium salt MTS into formazan by viable cells. Hence, the variation of the absorbance over the period of growth can be correlated to the cell growth profile. The results show that observed absorbance increased till 28 d, which further declined in all batches running for at least 35 d. The observation of cell viability and cell number variation with time define Monod’s growth model. The specific cell growth rate (µ) for each batch was calculated, using following equation:

\[ \mu = \frac{\ln X_t - \ln X_0}{t} \]

Where X_t and X_0 are varying values of cell concentration and t is the batch time.

The maximum specific growth rate for the chondrocytes was observed to be 0.001248 h⁻¹ and the generation time was calculated as ~555 h. The specific growth rates and the corresponding generation times of various batches are shown in Table 2.

The growth kinetics of chondrocytes culture was further evaluated as shown in Fig. 3. It shows that dx/dt (cell growth rate) initially increased up to 21 d of cultivation and later declined. Similarly, the kinetics of cell growth also show that the ratio of specific growth rate (µ) and maximum specific growth rate (µ_m) increased initially up to 21 d and later declined (Fig. 4). This resulted in sufficiently high number of viable cells...
with the growing age in exponential phase, as required for their therapeutic use.

Morphological Study

Usually, chondrocytes have a constant roundish shape and contain a nucleus, relatively large amount of clear cytoplasm and the common organelles. Initially, in the present study, cells showed round shape, which later changed during cell culture as a result of cell adhesion and spreading processes. After 1 wk of culture, flattened and spread chondrocyte cells with typical fibroblast morphology were seen (Fig. 5). Cells after 2 wk of culture under inverted microscope are shown in Fig. 6. For histological analysis, cells were stained with hematoxylin and eosin to differentiate the nuclei and cytoplasmic structures of the grown chondrocytes. The staining showed nuclei in blue and cytoplasm in pink colour.

Table 2—Growth rate and generation time

<table>
<thead>
<tr>
<th>Batch</th>
<th>Growth Rate (µ) (h⁻¹)</th>
<th>Generation Time (t,g) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>0.00121</td>
<td>545</td>
</tr>
<tr>
<td>2nd</td>
<td>0.00123</td>
<td>560</td>
</tr>
<tr>
<td>3rd</td>
<td>0.00133</td>
<td>520</td>
</tr>
<tr>
<td>4th</td>
<td>0.00128</td>
<td>541</td>
</tr>
<tr>
<td>5th</td>
<td>0.00119</td>
<td>580</td>
</tr>
<tr>
<td>6th</td>
<td>0.00145</td>
<td>478</td>
</tr>
<tr>
<td>7th</td>
<td>0.00139</td>
<td>499</td>
</tr>
</tbody>
</table>

Fig. 3—Plot of cell growth rate (dx/dt) vs time.

Fig. 4—Plot of µ/µmax vs time.

Fig. 5—Adhered chondrocyte cells with typical fibroblast morphology after 1 wk.

Fig. 6—Adhered chondrocyte cells with typical fibroblast morphology after 2 wk.

The positive staining indicated that the cells were in good morphological state. The stained chondrocyte cells at the age of 28 d are shown in Fig. 7.

Effect of Mechanical Agitation

Three batches of chondrocyte cultures were subjected to mild agitation and their growth pattern were compared with those not exposed to agitation and were in static flasks. The rate of increase of the cell count with increasing duration of incubation was greater in the cultures subjected to mild agitation as compared to the un-agitated cell cultures (Fig. 8). In static flasks, the cells were cultured with diffusion of
limited mass transfer of nutrients and gases and without hydrodynamic shear at tissue surfaces. In rotating vessel, the cells were dynamically suspended in a rotational fluid flow experiencing enhanced diffusion and decreased mass transfer limitation. This further suggests that mechanical stimulation may be mandatory for enhanced growth rate of chondrocytes. Studies similar to the present have also been conducted by other groups. Negri et al\textsuperscript{16} have demonstrated that human articular cartilaginous cells could be multiplied and grown on type I collagen substrate in a dynamic culture with the production of extracellular matrix. The transferred cells showed correct morphology, suitable differentiation and phenotype, and very good viability (~ 90%). In another study, Seoul et al\textsuperscript{17} showed the feasibility for regenerating cartilage tissue in vivo by implantation of chondrocyte cultured in vitro in a chemically defined serum free medium. The chondrocytes showed good growth within 28 d and good viability (> 90%), which is quite similar to the present study. Thus, such type of chondrocyte cultures can be used for repairing cartilaginous lesions.

**Conclusion**

The present study was carried out to optimize the growth conditions and process for the culture of chondrocytes from human donors and further ex vivo expansion of the chondrocyte cells in view of their therapeutic use. The growing cells were observed and evaluated for their viability and growth rate. The data related to growth rate ($\mu$) and generation time ($t_g$) enables the prediction of the growth profile of chondrocytes and facilitates scheduling the manufacturing of tissue-engineered cartilage applied for clinical treatment. To conclude, the present study demonstrates that the morphology and composition of chondrocyte cells can be modulated by flow conditions during in vitro cultivation. Further, the chondrocyte culture results optimized in this preliminary work could be a potential approach for large scale culture technique, which may be used for the treatment of cartilage degenerative lesions.

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