Pediatric auricular chondrocytes gene expression analysis in monolayer culture and engineered elastic cartilage

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KEYWORDS
Tissue engineering; Elastic cartilage; Auricular chondrocyte; Human fibrin; Gene expression analysis

Summary

Objectives: This study was aimed at regenerating autologous elastic cartilage for future use in pediatric ear reconstruction surgery. Specific attentions were to characterize pediatric auricular chondrocyte growth in a combination culture medium and to assess the possibility of elastic cartilage regeneration using human fibrin.

Study design: Laboratory experiment using human pediatric auricular chondrocytes.

Methods: Pediatric auricular chondrocytes growth kinetics and quantitative gene expression profile in three different types of media were compared in primary culture and subsequent three passages. Large-scale culture-expanded chondrocytes from the combination medium were then mixed with human fibrin for the formation of elastic cartilage via tissue engineering technique.

Results: The equal mixture of Ham’s F12 and Dulbecco’s Modified Eagle Medium (FD) promoted the best chondrocyte growth at every passage compared to the individual media. Chondrocytes differentiation index; ratio of type II to type I collagen gene expression level, aggrecan and elastin expression gradually decreased while passaging...
1. Introduction

Absences in vascularization and nerve endings in cartilage have limited its ability to remodel \[1,2\]. Autologous cartilage has always been the best candidate for reconstruction by means of repairing severe cartilage damages caused by trauma or aging related degeneration \[1\]. Autologous cartilage reduces the risk of transmitted diseases and immune rejection compared to the use of allograft and xenograft cartilage. However, limitations of donor cartilage, incorrect size and shape of the donor tissue and dissimilarity of the donor tissue to the native tissue have caused the congenital microtia repair to be delayed. Therefore, development in tissue engineering with the approach of creating grafts using patients’ own cells provide promising means of replacing deficient auricular cartilage \[3—6\].

For several years, tissue engineering has been studied as a possible alternative approach for autologous tissue grafting \[7—9\]. Many of the studies have proved that cartilage can be generated successfully both \textit{in vitro} and \textit{in vivo} by using various types of scaffold materials \[10—15\]. However, Lee et al. showed that polylactic-glycolic acid (PLGA) combined with cultured rabbit chondrocytes did not form cartilage when implanted as trachea substitute into rabbit \[16\]. The failure of the experiment in immunocompetent autologous model was due to foreign body inflammatory reaction against the synthetic scaffold material used to construct the engineered tissues \[13\]. Thus, a more suitable scaffold material needs to be established for further improvement of tissue engineering technology. Such material should also be easily available, not expensive and is a good natural biochemical compound with low risk of disease transmission.

Another crucial challenge in tissue engineering is to generate sufficient population of cells from a small amount of donor tissue. The limited number of isolated cells from donor tissue needs to be culture-expanded in order to obtain the required amount of cells to create large volume of tissue construct \[17\]. Isolated chondrocytes are usually cultured in basal medium that provides the basic nutrients needed. The medium is also supplemented with 10% serum and growth factors which are necessary for efficient cell number expansion in the shortest period of time \[18—21\]. Monolayer culture system has been recommended for human chondrocyte expansion \[22,23\] and the passaged chondrocytes were proven to retain the diploidy feature, hence are safe for cartilage regeneration \[24,25\]. However, cell expansion in monolayer system led to a problem in dedifferentiation due to failure in producing functional cartilaginous extracellular matrix (ECM) \[28\].

In previous studies, human auricular chondrocytes were cultured in Ham’s F12 medium for tissue engineering research \[24—27\]. It is known that culture medium plays an important role in supporting the chondrocytes proliferation rate and cell characteristic; yet comparison on the types of basal media which promote higher chondrocyte growth and preserve phenotype expression has not been reported. In this study, we focused on characterizing pediatric auricular chondrocyte growth kinetic and gene expression profile in three types of culture media for three passages. Medium which supported the best chondrocytes growth kinetic and provided sufficient number of cultured chondrocytes was used for tissue-engineered cartilage constructions. This study was also intended to investigate the likelihood of using fibrin derived from human plasma as a biomaterial for \textit{in vivo} tissue-engineered cartilage reconstructions. The ultimate goal for this study is to produce high quality engineered elastic cartilage which closely resembles the native elastic cartilage for future regeneration of an autologous elastic cartilage for ear reconstructive surgery.

2. Materials and methods

2.1. Chondrocytes isolation and culture

This study was approved by the Research & Ethical committee of the Faculty of Medicine, National University of Malaysia. All samples of pediatric but they were then restored in engineered tissues after implantation. The engineered cartilage was glistening white in color and firm in consistency. Histological evaluation, immunohistochemistry analysis and quantitative gene expression assessment demonstrated that the engineered cartilage resemble the features of native elastic cartilage.

\textbf{Conclusion:} Pediatric auricular chondrocytes proliferate better in the combination medium (FD) and the utilization of human fibrin as a biomaterial hold promises for the regeneration of an autologous elastic cartilage for future application in ear reconstructive surgery.
auricular cartilage (n = 6) were obtained from excessive tissues discarded from otologic surgical procedures (Table 1). The samples were minced and digested with 0.6% type II Collagenase (Gibco, Grand Island, NY, USA) at 37 °C for 12 h to isolate the chondrocytes. Total cell yield was then quantified using haemocytometer (Weber Scientific International, Ltd., Middlex, England) and cell viability was determined by trypan blue dye exclusion test (Gibco, Grand Island, NY, USA). Isolated chondrocytes were then seeded in 6 well plates (Falcon, Franklin Lakes, NJ) with initial seeding of 5000 cells/cm² in three different types of basic medium: (1) Ham’s F12 Nutrient Mix Medium (F12, Gibco, Grand Island, NY, USA), (2) Dulbecco’s Modified Eagle Medium (DMEM, Gibco, Grand Island, NY, USA) and (3) equal volume mixture of F12 and DMEM (FD; 1:1 ratio) supplemented with 10% fetal bovine serum (FBS, Gibco, Grand Island, NY, USA). All cultures were maintained in 5% CO₂ incubator (Jouan, Duguay Trouin, SH) at 37 °C with the media changed twice per week. Upon confluence, primary culture, P0 was trypsinized with 0.05% trypsin-EDTA (Gibco, Grand Island, NY, USA) and passaged three times (P1, P2, P3). Chondrocytes growth rates (cells/(day cm²)) and total number of cell doubling were calculated and recorded at every passage.

### 2.2. Quantitative gene expression analysis by real-time PCR

Total RNA of cultured chondrocytes at serial passages were extracted using TRI reagent (Molecular Research Center, Cincinnati, OH) to evaluate the changes in gene expression during cell expansion. Total RNA was extracted according to the manufacturer’s instructions. Yield and purity of the extracted RNA was determined by spectrophotometer (Bio-Rad, Hercules, CA). Auricular chondrocytes differentiation (type II collagen, elastin and aggrecan core protein), dedifferentiation (type I collagen) and hypertrophy (type X collagen) gene expression profile were quantified with real-time PCR technique. Primers for human GAPDH, type I, II, X collagen, elastin and aggrecan core protein were designed with Primer 3 software based on the GeneBank database sequences corresponding to the specific gene Accession Number as stated in Table 2. The reaction kinetic of each primer set was verified with standard curve (Ct value versus serial dilution of total RNA) and melting profile. Real-time PCR reaction was performed with 100 ng of total RNA, 400 nM of each primer and iScript One-Step RT-PCR kit with SYBR Green (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Reactions were carried out using Bio-Rad iCycler with profile of; cDNA synthesis for 30 min at 50 °C; pre-denaturation for 2 min at 94 °C; PCR amplification for 38 cycles with 30 s at 94 °C, 30 s at 60 °C and 30 s at 72 °C. This series of cycles was followed by melt curve analysis to check the reaction specificity. Expression level of each targeted gene was normalized to GAPDH and chondrocyte differentiation index (the ratio of type II to type I collagen gene expression level; CII/CI index) was then calculated for statistical analysis.

### Table 1 Sample of cartilage obtained from six pediatric patients

<table>
<thead>
<tr>
<th>Sample</th>
<th>Age</th>
<th>Sex</th>
<th>Type of surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>Female</td>
<td>Mastoid exploration</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>Male</td>
<td>Mastoid exploration</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>Female</td>
<td>Mastoid exploration</td>
</tr>
<tr>
<td>4</td>
<td>9</td>
<td>Male</td>
<td>Mastoid exploration</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>Male</td>
<td>Myringoplasty</td>
</tr>
<tr>
<td>6</td>
<td>12</td>
<td>Female</td>
<td>Myringoplasty</td>
</tr>
</tbody>
</table>

### Table 2 Primer sequences used in real-time PCR for quantitative gene expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession no.</th>
<th>Primer 5’ → 3’</th>
<th>PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH [43]</td>
<td>BC020308</td>
<td>F: tcc ctg agc tga acg gga ag</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: gga gga gtg gtc gct gt</td>
<td>217</td>
</tr>
<tr>
<td>Type I collagen [44]</td>
<td>NM_000088</td>
<td>F: agg gct cca acg aga tgg aag</td>
<td>222</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: tac agg aag cag aca gaa cca</td>
<td></td>
</tr>
<tr>
<td>Type II collagen [45]</td>
<td>NM_001844</td>
<td>F: cta tct gga cga agc agc tgg</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: atg ggt gca atg tca atg atg</td>
<td></td>
</tr>
<tr>
<td>Type X collagen [46]</td>
<td>NM_000493</td>
<td>F: agc cag ggt tgc cag cag ca</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: tt tcc cac tcc agg agg gc</td>
<td>385</td>
</tr>
<tr>
<td>Elastin [47]</td>
<td>NM_00501</td>
<td>F: ggc ctg gag gca aac ctc tt</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: cca cca act cct ggg aca cc</td>
<td>189</td>
</tr>
<tr>
<td>Aggrecan core protein [48]</td>
<td>NM_001135</td>
<td>F: cac tgt tac cgc cac ttc cc</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: acc agg gga agt ccc ctt cg</td>
<td>183</td>
</tr>
</tbody>
</table>
2.3. Large-scale chondrocyte culture and tissue-engineered pediatric elastic cartilage with human fibrin

Chondrocytes were plated in 175 cm² culture flasks (Falcon, Franklin Lakes, NJ) at a density of 5000 cells/cm². Confluence cells were then harvested by trypsinization and suspended into human plasma at cell density of 3 x 10⁶ cells/ml. Human plasma was collected from healthy donors using sodium citrate blood collection tubes (Greiner Bio-one, Austria). The resulted admixer (Chondrocytes in plasma) was then polymerized by adding 25 μl of CaCl₂ (1 M)/ml of plasma used. The chondrocytes-fibrin constructs were maintained in CO₂ incubator for 1 week before being implanted subcutaneously at the dorsal part of the nude mice under general anesthesia (ketamine, xylazil and zoletil). Care of the nude mice was carried out under the animal facility guideline of the Animal Unit, Institute of Medical Research Malaysia.

2.4. Tissue-engineered pediatric elastic cartilage evaluation

After 8 weeks of implantations, the constructs were harvested for macroscopic observation, histological analysis and quantitative gene expression evaluation. The tissue-engineered cartilage was divided into two equal half with one part fixed in 10% phosphate buffered formalin (Fisher Scientific, Fair Lawn, NJ) and processed into paraffin embedded block. Another half of the excised tissue was digested with collagenase enzyme and total RNA was extracted for quantitative gene expression analysis as mentioned earlier. In standard histological evaluation, paraffin blocks were sectioned and the slides sections were stained with Hematoxylin & Eosin (H&E staining), Safranin O staining and Verhoeff’s staining. For immunohistochemistry evaluation, the staining protocol was carried out using the EnVision Detection kit (DAKO, Glostrup, Denmark). Briefly, tissue sections were treated with peroxidase block for 10 min prior to incubation with antibody. Antibodies used in this study were mouse antihuman Collagen type I, mouse antihuman Collagen type II and mouse antihuman Fibroblast Surface Protein (Sigma, St. Louis, MO). After incubation with antibody, tissue sections were applied with anti-mouse peroxidase polymer and incubated for 15 min. After washing, the tissues were then treated with the freshly prepared 3,3′-diaminobenzidine (DAB, DAKO) substrate for color development. Native human auricular cartilage was used as normal control for comparison.

2.5. Statistical analysis

Data for chondrocytes growth rate and total number of cell doubling in each medium at every passage (P0, P1, P2 and P3); expression level of each targeted gene (Collagen types I, II and X, aggrecan, elastin) and chondrocyte differentiation index were collected from six samples. Values were presented as mean ± standard error of mean (S.E.M.). Student’s t-test was used to compare data between groups. Differences at 5% level were considered significant.

3. Results

3.1. Samples of cartilage

Cartilage samples were obtained from patients with the age range from 7 to 12 years old. There was a balanced ratio of male to female patients. Cartilage was attained from excessive specimen after mastoid exploration or myringoplasty surgery (Table 1).
3.2. Cultured chondrocytes morphologic features

Chondrocytes in primary culture (P0) reached confluence within 2—3 weeks and remained polygonal in shape in all three types of culture media (Fig. 1A). As the cultures were passaged, chondrocytes changed to be more elongated in shape and exhibited spindle-like feature at the end of passage 3 (Fig. 1B). Cultured chondrocytes in all three tested media (F12, DMEM and FD) demonstrated the same morphologic changes resulted from passaging.

3.3. Growth kinetic of cultured pediatric auricular chondrocytes

The combination medium (FD) promotes higher chondrocyte growth rate in every passage in comparison to cells cultured in F12 or DMEM alone. The auricular chondrocytes scored the lowest growth rate when grew in F12 medium (Fig. 2). Growth rate of the cultured chondrocytes in F12 was almost half (P0) or less than half (P1, P2 and P3) when compared to FD (Fig. 2). Chondrocyte growth rate in FD was significantly higher than F12 in all passages but only significantly higher at P3 when compared to DMEM (p < 0.05; Fig. 2). Chondrocytes viability was greater than 94% at every passage whereby no significant difference exists between the three culture media. Total number of cell doubling (TCD) which indicates the magnitude of chondrocytes expansion in the culture showed chondrocytes that were cultured in FD scored the highest TCD (10.61 or 1563 times increase in cell number) compared to F12 (7.83 or 228 times increase in cell number) and DMEM (9.32 or 639 times increase in cell number).

3.4. Quantitative gene expression analysis on cultured pediatric auricular chondrocytes

In monolayer culture of the auricular chondrocytes, the gene expression level of type II collagen gradually reduced throughout passaging, where as, type I collagen gene expression level gradually increased throughout passaging. Both factors had contributed to the declined of CII/CI index from primary culture (P0) to passage 3 (P3) in all three type of tested media (Table 3). Cultured chondrocytes in F12 scored the highest CII/CI index in all passages, followed by the cells in FD (Table 3). CII/CI index of chondrocytes in F12 was significantly higher than DMEM at P0, P1 and P2 but only significant higher than FD at P0 and P1 (Table 3). Quantitative gene expression assessment for aggrecan core protein in monolayer culture of pediatric auricular chondrocytes showed both F12 and FD media scored higher expression compared to DMEM at all passages (Table 4). Aggrecan core protein gene expression level in F12 and FD were only significantly higher than DMEM at P3 (Table 4). Pediatric auricular chondrocytes cultured in FD scored higher elastin gene

![Fig. 2](image)

**Table 3** Ratio of type II to type I collagen gene expression levels (CII/CI index) in monolayer culture of pediatric auricular chondrocytes (mean of six different specimens ± S.E.M.)

<table>
<thead>
<tr>
<th>Type of medium</th>
<th>Ham’s F12 Nutrient Mix Medium (F12)</th>
<th>Dulbecco’s Modified Eagle Medium (DMEM)</th>
<th>Equal mixture of F12 and DMEM (FD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary culture (P0)</td>
<td>1.6621 ± 0.42221*</td>
<td>0.2251 ± 0.0583</td>
<td>0.7570 ± 0.2076</td>
</tr>
<tr>
<td>Passage 1 (P1)</td>
<td>0.1576 ± 0.04871*</td>
<td>0.0189 ± 0.0047</td>
<td>0.0437 ± 0.0125</td>
</tr>
<tr>
<td>Passage 2 (P2)</td>
<td>0.0082 ± 0.0033*</td>
<td>0.0015 ± 0.0005</td>
<td>0.0059 ± 0.0033</td>
</tr>
<tr>
<td>Passage 3 (P3)</td>
<td>0.0005 ± 0.0002</td>
<td>0.0002 ± 0.0001</td>
<td>0.0003 ± 0.0001</td>
</tr>
</tbody>
</table>

CII/CI index in F12 was significantly higher than DMEM (*); FD (†), p < 0.05.

FD CII/CI index in FD was significantly higher than DMEM, p < 0.05.
expression level at P1, P2 and P3 when compared to F12 and DMEM (Table 5). However, significant difference of elastin gene expression level was only seen at P3 when FD was compared with DMEM (Table 5). Monolayer cultured auricular chondrocytes in all experimental groups scored very low expression level of type X collagen gene (hypertrophy marker).

3.5. Evaluation of tissue-engineered pediatric elastic cartilage

In vitro chondrocytes-fibrin constructs after stabilization for 1 week demonstrated a stable three-dimensional structure, which can be easily transferred with surgical forceps during implantation (Fig. 3A). After 8 weeks of implantation, the

[Table 4 Aggrecan core protein gene expression in monolayer culture of pediatric auricular chondrocytes normalized to GAPDH (mean of six different specimens ± S.E.M.)]

<table>
<thead>
<tr>
<th>Type of medium</th>
<th>Ham’s F12 Nutrient Mix Medium (F12)</th>
<th>Dulbecco’s Modified Eagle Medium (DMEM)</th>
<th>Equal mixture of F12 and DMEM (FD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary culture (P0)</td>
<td>0.0454 ± 0.0064</td>
<td>0.0260 ± 0.0069</td>
<td>0.0342 ± 0.0094</td>
</tr>
<tr>
<td>Passage 1 (P1)</td>
<td>0.0069 ± 0.0007</td>
<td>0.0054 ± 0.0012</td>
<td>0.0086 ± 0.0018</td>
</tr>
<tr>
<td>Passage 2 (P2)</td>
<td>0.0030 ± 0.0004</td>
<td>0.0022 ± 0.0005</td>
<td>0.0046 ± 0.0008</td>
</tr>
<tr>
<td>Passage 3 (P3)</td>
<td>0.0013 ± 0.0002*</td>
<td>0.0005 ± 0.0001</td>
<td>0.0018 ± 0.0002*</td>
</tr>
</tbody>
</table>

*Aggrecan core protein gene expression in F12 was significantly higher than DMEM, p < 0.05.

[Table 5 Elastin gene expression in monolayer culture of pediatric auricular chondrocytes normalized to GAPDH (mean of six different specimens ± S.E.M.)]

<table>
<thead>
<tr>
<th>Type of medium</th>
<th>Ham’s F12 Nutrient Mix Medium (F12)</th>
<th>Dulbecco’s Modified Eagle Medium (DMEM)</th>
<th>Equal mixture of F12 and DMEM (FD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary culture (P0)</td>
<td>0.0318 ± 0.0040</td>
<td>0.0458 ± 0.0082</td>
<td>0.0457 ± 0.0144</td>
</tr>
<tr>
<td>Passage 1 (P1)</td>
<td>0.0116 ± 0.0016</td>
<td>0.0172 ± 0.0042</td>
<td>0.0219 ± 0.0073</td>
</tr>
<tr>
<td>Passage 2 (P2)</td>
<td>0.0066 ± 0.0006</td>
<td>0.0060 ± 0.0023</td>
<td>0.0172 ± 0.0067</td>
</tr>
<tr>
<td>Passage 3 (P3)</td>
<td>0.0046 ± 0.0007</td>
<td>0.0025 ± 0.0011</td>
<td>0.0095 ± 0.0029 (^\uparrow)</td>
</tr>
</tbody>
</table>

\(^\uparrow\)Elastin gene expression in FD was significantly higher than DMEM, p < 0.05.

Fig. 3 Tissue-engineered construct before and after implantation. (A) One week in vitro constructs demonstrated a stable three-dimensional structure. (B) After 8 weeks of implantation, the construct was firm and resistance to compression.
engineered tissues were whitish and glistening in appearance (Fig. 3B). When the tissues-engineered cartilage was palpated by forceps, it was firm and resistance to compression. In histological evaluation, Safranin O stain showed positive orange-red staining, denoting abundant proteoglycans production in the tissues-engineered cartilage (Fig. 4A). A fibrous capsule resembling perichondrium at the edge of the tissues-engineered cartilage was also noted in the micrograph (Fig. 4A). The Hematoxylin & Eosin stain demonstrated round to oval lacunae cells embedded in a basophilic matrix (Fig. 4B). Verhoeff’s staining showed black-blue fiber network in the tissue sections, indicating the presence of elastin fiber (Fig. 4C). Immunohistochemical analysis with type II collagen antibody was strongly positive, demonstrating the tissues-engineered cartilages were rich in type II collagen (Fig. 5A). The tissue sections also stained faintly with type I collagen antibody (Fig. 5B) and no positive staining was detected with fibroblast surface protein antibody (Fig. 5C). Quantitative gene expression evaluation on tissue-engineered cartilage demonstrated significantly higher expression level of both type II and type I collagen gene compared to native pediatric elastic cartilage (Table 6). However, native cartilage scored significantly higher CII/CI index compared to

<table>
<thead>
<tr>
<th></th>
<th>Type I collagen</th>
<th>Type II collagen</th>
<th>CII/C I Index</th>
<th>Aggrecan core protein</th>
<th>Elastin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Native</strong></td>
<td>0.0027 ± 0.0005</td>
<td>0.3885 ± 0.1892</td>
<td>88.9636 ± 26.1908</td>
<td>0.2003 ± 0.0475</td>
<td>0.1648 ± 0.0346</td>
</tr>
<tr>
<td><strong>Tissue-engineered</strong></td>
<td>0.1532 ± 0.0537</td>
<td>1.9523 ± 0.1454</td>
<td>24.1421 ± 7.3301</td>
<td>0.1986 ± 0.0422</td>
<td>0.1954 ± 0.0460</td>
</tr>
</tbody>
</table>

# Significant differences in gene expression level between native cartilage compared to tissue-engineered cartilage, \( p < 0.05 \).
tissue-engineered cartilage (Table 6). Both aggrecan core protein and elastin expression level in tissue-engineered cartilage were comparable to native pediatric elastic cartilage (Table 6). Type X collagen gene expression was very low in both tissue-engineered cartilage and native cartilage.

4. Discussion

In this study, we have shown that viable and proliferative auricular chondrocytes can be successfully isolated from pediatric ear cartilage obtained from otologic surgery. During monolayer culture expansion, the chondrocytes changed from polygonal shape in the early passage to fibroblastic or spindle-like appearance at the later passage (P3) regardless of the type of medium used. These morphological changes were previously reported in other human cell culture studies [29–31], which are caused by the modifications of cell cytoskeleton in the monolayer culture [32,33]. The quantitative gene expression results in this study have been shown to correlate with this dedifferentiation phenomenon; demonstrating the gradual reduction of chondrocyte differentiation CII/CI index through passaging. Previous study has suggested that a three-dimensional environment will promote the chondrocytes to redifferentiate [23]. Quantitative gene expression results demonstrated that the CII/CI index, aggrecan core protein and elastin gene expression of tissue-engineered cartilage are similar to the native elastic cartilage gene expression level after 8 weeks of in vivo implantation [34].

Although FD supported lower chondrocyte differentiation CII/CI index compared to F12 during monolayer expansion, the cultured chondrocytes proliferated twice faster in FD compared to F12. Moreover, FD promoted the highest chondrocyte doubling amongst tested media. Thus, FD ensures bigger harvest in shorter period of time for the purpose of cartilage regeneration. Furthermore, FD demonstrated higher expression of aggrecan core protein and elastin genes in which both are important extracellular matrix components for the functionality of elastic cartilage. Most of the previous studies on elastic cartilage tissue engineering in either animal model [13] or human auricular chondrocytes monolayer culture expansion [24–27] were based on F12 medium. The results in this study strongly suggested that FD should be considered for future study in elastic cartilage tissue engineering compared to F12 or DMEM alone.

Subsequently, cultured chondrocytes in FD mixed well with human fibrin resulting in the polymerization of fibrinogen in the plasma and formed a physiologically stable construct for transplantation. This technique utilizes the natural blood clotting elements to form the three dimensional scaffold for chondrocytes attachment. Fibrin gel has been well known to be biodegradable and biocompatible. It also allows initial immobilization, therefore cell loss has been minimized and the cells distribute homogeneously [35]. Furthermore, native cartilage is better of with continuous supply of nutrient via homeostasis, while tissue-engineered cartilage lacks of biomechanical signals and has to depend only to the passive diffusion of nutrient [36]. For this reason, we have integrated fibrin as the scaffold in which chondrocytes are able to proliferate and differentiate. High content of fibronectin in fibrin glue is believed to facilitate the cell-matrix interactions. Besides that, fibrin glue is highly porous with an interconnected pore network that would help to deliver essential nutrient and extracellular matrix component for better cell growth [37]. In addition to that, in our experiment autologous human fibrin that was obtained from patient’s plasma has been able to reduce the cost. Furthermore, this method enables the use of autologous plasma, thus eradicates disease transmission and eliminates the risk of foreign body immune rejection using synthetic scaffold materials [38,39].

Chia et al. suspended the dedifferentiate cells in alginate and found that alginate has stimulated deposition of higher glycosaminoglycans and collagen type II and believed that this came from re-expression of chondrocyte phenotype and the maintenance of the morphological properties of chondrocytes [40]. However, fairly high amount of type I collagen gene was expressed in the tissue-engineered cartilage and caused lower differentiation CII/CI index when compared to the native elastic cartilage. This may be caused by the implanted cultured chondrocytes undergoing the process of development and cartilage remodeling towards becoming mature cartilage; since previous study has reported the production of type I collagen in immature cartilage [41]. The histological staining showed that the tissue-engineered cartilage exhibited architectural characteristics of a functional elastic cartilage. The well-distributed lacunae chondrocytes was found to embed within cartilage proteoglycans ground substance and enclosed by abundant elastic fibers. The immunohistochemical analysis also proved that the tissue-engineered cartilage did not invade or mix with fibroblast. Furthermore, quantitative gene expression on type X collagen verified that cultured chondrocytes did not have the tendency to develop into osteoblast and the tissue-engineered cartilage was not calcified.
5. Conclusions

This study has demonstrated the advantages of equal volume mixture of F12 and DMEM as the culture medium compared to the individual medium in promoting pediatric auricular chondrocytes proliferation and tissue-engineered elastic cartilage regeneration. This study has proven a method for cartilage tissue engineering using the low cost, easily available and highly safe human fibrin as the biomaterial. This is in line with the work using autologous serum for chondrocyte culture [42]. With further investigations, we strongly believe that the reconstruction of a reliable tissue-engineered human auricular cartilage for congenital microtia reconstruction in pediatrics will be a reality.

Conflict of interest statement

We wish to confirm that there are no known conflicts of interest associated with this manuscript publication and there has been no significant financial support for this work that could have influenced its outcome.

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