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The Expansion Potential of Human Nasal Septum Chondrocytes for the Formation of Engineered Cartilage

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ABSTRACT: Recent development in tissue engineering has enabled a new approach in tissue repair and reconstructive surgery using patient's own cells. Human nasal septum cartilage is an optimum candidate as a starting material for this purpose. It is essential to understand the basic requirements for monolayer culture expansion of human nasal septum chondrocytes before using it in tissue engineering. The objective of this study was to investigate the effects of different media; Ham's F12 Medium, Dulbecco's Modified Eagle Medium (DMEM) or the equal combination of both media (F12:DMEM) on monolayer culture expansion of human nasal septum chondrocytes. A hydrogel, Pluronic F-127, was used as a biomaterial for cartilage reconstruction in nude mice with culture expanded chondrocytes. We demonstrated that F12:DMEM in 1:1 ratio promoted the highest human nasal septum chondrocytes proliferation and provided sufficient numbers of chondrocytes for cartilage engineering. This medium was effective for culture expansion to more than 2000 folds within 8 weeks. Although none of the medium maintained the chondrocytes' phenotype in long-term culture, the dedifferentiated cultured chondrocytes re-express collagen type II gene after being reconstructed into engineered tissue with Pluronic F-127. This hydrogel provided a 3-dimensional environment for cultured chondrocytes to regain their chondrocytic phenotype and thus initiate the generation of a good quality engineered cartilage.

KEYWORDS: Human Chondrocyte, Tissue Engineering, Cartilage, Hydrogel.

INTRODUCTION

Cartilage is often needed for reconstructive surgery, such as for defects of the ear, nose and trachea[1]. Autograft and allograft cartilage are also commonly used for the repair of osteochondral lesions[2]. Since the use of allograft and xenograft cartilage possess the potential risks of immune rejection and infectious disease transmission, autologous cartilage offers an alternative solution for the purpose. Unfortunately, using autologous cartilage means only a limited amount of cartilage can be harvested without causing deformity and morbidity to the donor sites.

Through tissue engineering technology, freshly isolated human costal chondrocytes from a patient's own tissue was able to be used to construct human nose[3]. Achievements in recent years have shown the possibility of forming different human shape cartilage, such as nasal tip[4], ear[5], trachea[6], phalanges and small joints[7]. This was achieved by using freshly isolated chondrocytes or short-term cultured chondrocytes on the pre-shaped scaffold materials.

Human nasal septum cartilage can be opted as a starting material for tissue engineering since it is high in cell density with excellent proliferation capacity and furthermore, may also be isolated from a broad range of age[8,9]. However, freshly isolated chondrocytes or short-term cultured chondrocytes with a small number of cells have limited application to the repair of a clinical -size defect, because a lot of chondrocytes are needed for this purpose[10]. Since only a finite amount of cartilage may be harvested without inducing morbidity at the donor site, the limited numbers of isolated chondrocytes need to be culture-expanded first into a large number *in vitro* before engineered tissue can be reconstructed.

Studies on the culture expansion potential of human nasal septum chondrocytes are still limited. Most of the *in vitro* studies evaluated the expansion potential of the nasal septum chondrocytes in short-term culture without them undergoing any passages[8,11]. The most commonly used medium is Dulbecco's Modified Eagle Medium (DMEM)[8,11]. Dunham & Koch (1998) used DMEM to culture-expanded human septal chondrocytes until passage 4 for their study on growth factor effects in a serum free environment[12]. On the other hand, Ham's

F12 medium was widely used in animal chondrocytes[6,13] and human auricular[14], as well as articular[15] chondrocytes, culture expansion. There have been reports regarding Ham's F12 and DMEM combination medium usage in human bone marrow[16,17], articular[18,30] and auricular[19,30] chondrocytes expansion. However, a comparison of which type of medium promotes optimal cell expansion for human nasal septum chondrocytes *in vitro* has yet to be reported.

Human nasal septum chondrocytes have already been shown to incorporate well with biodegradable and non-biodegradable pre-shaped scaffold materials[14,18-19]. However, Lee et al. (2002) showed that polylactic-glicolic acid (PLGA) with cultured rabbit chondrocytes was unable to form cartilage when implanted as tracheal substitute into rabbits[6]. Homicz et al. (2003) commented that human nasal septum chondrocytes cultured in alginate maintained better chondrocytic phenotype by producing more sulfated glycosaminoglycan than when seeded onto polyglicolic acid (PGA)[11]. In a different study, biodegradable, the biocompatible hydrogel Pluronic F-127, which is a mixture of 30% polypropylene and 70% polyethene was claimed to be a better biomaterial compared to PGA and alginate, especially in preventing inflammatory response in immunocompetent animal models[19]. Further studies of using this biomaterial showed great success in generating engineered human nasal septum cartilage[14] and tracheal reconstruction[20] in nude mice models, and also autologous ear helix cartilage in a large animal model[26].

Our research was aimed to evaluate the effects of HAM's F12 medium, Dulbecco's Modified Eagle Medium and the combination of both media in an equal ratio (1:1) on the proliferation and phenotype expression of human nasal septum chondrocytes. The type of medium which supported the highest chondrocytes proliferation was used for engineered cartilage formation.

Materials and Methods

Human Nasal Septum Harvest

Nasal septum cartilage samples were harvested during elective septoplasty from 9 consenting patients aged from 20 to 60 years. These specimens were redundant tissues and their use in this study was approved by the Research and Ethical Committee of the Medical Faculty, Universiti Kebangsaan Malaysia. Removed cartilage were placed in sterile normal saline at 4°C and transported to the Tissue Engineering Laboratory, Hospital Universiti Kebangsaan Malaysia.

Nasal Septum Chondrocyte Isolation

Each cartilage sample was weighed and stripped of its perichondrium, minced into small pieces (1 mm^3), washed with phosphate buffer saline (PBS; pH 7.2; Gibco, Grand Island, NY) containing 100 U/mL of penicillin, 100 µg/mL of streptomysin and 0.25 µg/mL of amphotericin B (Gibco) and digested with 0.6% collagenase II (Gibco) in an orbital incubator shaker (Stuart Scientific, Redhill, UK) at 37°C for 12 hours. Suspension containing isolated chondrocytes were then centrifuged at 6,000 rpm for 5 minutes. The resulting cell pellet was washed twice with PBS to remove remaining digestive enzyme. After the final centrifugation, the cell pellet was re-suspended in PBS for a total cell count with a hemacytometer (Weber Scientific International Ltd. Middlx, England) and cell viability was determined using trypan blue dye exclusion test (Gibco).

Chondrocytes *in vitro* Culture

Freshly isolated chondrocytes were then seeded in six-well tissue culture plates (Falcon, Franklin Lakes, NJ) as the primary culture (P0) at a density of 5,000 cells/cm^2 in three different media: HAM's F12 medium (F12; Gibco, Grand Island, NY), Dulbecco's Modified Eagle Medium (DMEM; Gibco); and a combination of Ham's F12 medium and Dulbecco's Modified Eagle Medium (F12:DMEM)(1:1). Each medium was supplemented with 10% fetal bovine serum (FBS; Gibco), 200 mM L-glutamine (Gibco), 100 U/mL of penicillin (Gibco), 100 µg/mL of streptomysin (Gibco), 0.25 µg/mL of amphotericin B (Gibco) and 50 µg/mL of ascorbic acid (Sigma, St. Louis. MO). All cultures were maintained in a 5% CO_2 incubator (Jouan, Duguay Trouin, SH) at 37°C with the culture medium changed twice a week. When the primary culture (P0) reached confluence, it was trypsinized with 0.05% trypsin-EDTA (Gibco). The harvested cultured chondrocytes were washed three times in PBS (Gibco). The resulting cell pellet was then resuspended in 10 mL of PBS for a total cell count and cell viability. Cultured chondrocytes were then sub-cultured (passage 1; P1) into a new six-well tissue culture plate (Falcon) with the same type of medium and culture environment as previously described. Once confluent, it was sub-cultured for another two passages (P2 and P3). Total cell number and cell viability were recorded at every passage.

Chondrocytes Culture Characterization

Cell morphologic features in the culture were examined everyday by using an inverted light microscope (Olympus, Shinjuku-ku, Tokyo). Growth rate of cultured chondrocytes in each medium at every passage (P0, P1, P2 and P3) was calculated as the average increase of chondrocyte number in one day/cm^2. The population doubling time (days) and total number of cell doublings were calculated in each medium at every passage.

Total RNA Extraction

Total RNA from the primary culture (P0) and each

passage (P1, P2 and P3) was extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instructions. Polyacryl Carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA. The RNA pellet was then washed with 75% ethanol and dried before it was dissolved in RNase and DNase free distilled water (Invitrogen, Carlsbad, CA). The yield and purity of the isolated RNA was assessed by spectrophotometer (Bio-Rad, Hercules, CA). Total RNA obtained was stored at -80°C immediately after extraction.

One Step RT-PCR

Expression of type I and type II collagen genes were evaluated by one-step reverse transcriptase-polymerase chain reaction (One Step RT-PCR; Invitrogen, Carlsbad, CA). Expression of human β-actin gene was used as a control. The specific sense and antisense primers used for the reaction were designed from the sequence listed in the NIH GenBank database and had the following sequences: type I collagen, 5′-AAGGCTTCCAAGGTCCCCCTGGTG-3′ and 5′-CAGCACCAGTAGCACCATC- ATTTC-3′; type II collagen, 5′-CTGGCAAAGA-TGGTGAGACAGGTG-3′ and 5′-GACCAT- CAGTGCCAGGAGTGC-3′; β-actin, 5′-CC-GGCTTCGCGGGCGACG-3′ and 5′-TCCCGG-CCAGCCAGGTCC-3′. One Step RT-PCR reaction mix was prepared according to the manufacturer's instructions. Each reaction consisted of 100 ng total RNA and 10 pmol each of sense and antisense primers. One step RT-PCR was performed in a 9700 thermal cycler (Perkin Elmer, Norwalk, CT) with a reaction profile of: cDNA synthesis for 30 min at 50°C; pre-denaturation for 2 min at 94°C; and PCR amplification for 38 cycles with 30 sec at 94°C, 30 sec at 60°C and 1 min at 72°C. This series of cycles were followed by a final extension of 72°C for 2 min. Subsequently, the PCR products were electrophoresed on a 1.5% agarose gel (Invitrogen, Carlsbad, CA) stained with ethidium bromide (Sigma, St. Loius, MO) and visualized by UV transillumination (Vilber Lourmat, Marne La Vallee, France).

Large-scale Chondrocyte Culture for Cartilage Tissue Engineering

A total of 3.5×10^6 cultured chondrocytes were plated in four 175cm^2 culture flasks (Falcon) at a density of 5,000 cells/cm^2. Once culture reached confluence, chondrocytes were trypsinized with 0.05% trypsin-EDTA (Gibco) and then harvested. The culture-expanded chondrocytes were then mixed and suspended in hydrogel for engineered cartilage formation.

Cartilage Tissue Engineering

Cultured chondrocytes were suspended in a 30% (wt/vol) solution of a co-polymer of polyethylene oxide and polypropylene oxide, Pluronic F-127 (BASF, Mount Olive, NJ) at a cell density of 3×10^7 cells/mL at 4°C . The resulting admixer (0.4ml) was then injected subcutaneously on the dorsal part of an 8-week old nude mice (right side of the lumbar area) under general anesthesia. The same volume of pluronic F-127 without cells was injected on the left side as a blank control. The engineered tissues were harvested after 8 weeks of *in vivo* implantation. The nude mice were sacrificed by anesthetic overdose and the engineered tissues formed were carefully dissected free from surrounding soft tissue.

Engineered Cartilage Evaluation

After the engineered tissue was removed from the nude mice, it was divided into two equal halves with one part fixed in 10% phosphate buffered formalin (Fisher Scientific, Fair Lawn, NJ) and was later processed and embedded in paraffin blocks for histological analysis. Slides sections were stained with hematoxylin & eosin (H&E staining) and Safranin O staining. Another half of the excised tissue was digested with collagenase enzyme and total RNA was extracted from the isolated cells. Gene expression of the cells isolated from the engineered cartilage was evaluated by one-step RT-PCR technique as mentioned earlier.

Statistical Analysis

Data for cell viability, growth rate, population doubling time and total number of cell doublings in each medium at every passage (P0, P1, P2 and P3) were collected from nine samples. Values were presented as mean ± standard error of mean (SEM). Student's *t* test was used to compare data between groups. Differences at 5% level were considered significant.

Results

Cartilage Harvest and Chondrocytes Isolation

The average wet weight of the nasal septum cartilage was 200 ± 54 mg. The average chondrocytes yield was $2.1 \times 10^3 \pm 0.7 \times 10^3$ cells per mg of cartilage. Viability of the isolated chondrocytes was 90.6 ± 2.3%. There was no correlation between chondrocytes yield and viability with the age of donor.

Human Nasal Septum Chondrocytes Morphologic Features in Monolayer Culture

Freshly isolated chondrocytes adhered fast onto the tissue culture plate in Ham's F12 medium. Cell multiplication started on the third day and became prominent after 1 week of culture. Chondrocytes appeared uniform in size with polygonal shape and distributed as islet of growth (Fig. 1A). Chondrocytes in primary culture (P0) reached confluent within 3 weeks and remained polygonal in shape. As the cultured chondrocytes were passaged, the shape become elongated.

At this stage, chondrocytes grew slower and took longer time to reach confluence. At passage 3 (P3), cultured chondrocytes were spindle shaped and seemed bigger in size compared to the primary culture (Fig. 1D). Chondrocytes at P3 were very slow in proliferation and only reached sub-confluence at the end of 3 weeks culture.

Both freshly isolated and cultured chondrocytes adhered slower onto the tissue culture plate in Dulbecco's Modified Eagle Medium (DMEM) compared to Ham's F12 medium. The cells only managed to adhere fully after the fourth day of culture. Cell multiplication was evident after one week of culture. In primary culture (P0), chondrocytes grown in DMEM medium appeared uniform in size but with more elongated shape than chondrocytes cultured in Ham's F12 medium (Fig 1B). The cultured chondrocytes became more elongated as they underwent passages. At passage 3 (P3), the chondrocytes appeared spindle shaped and bigger in size (Fig 1E). Chondrocyte growth was indeed very slow at P3 and only reached sub-confluence at the end of third week culture.

Chondrocytes cultured in an equal volume mix of Ham's F12 medium and Dulbecco's Modified Eagle Medium (F12:DMEM) adhered and multiplied faster in the tissue culture plate and, thus, produced higher cell density by the second week of culture compared to either of the individual media (Fig. 1C). The morphology of the cultured chondrocytes in F12:DMEM appeared similar to that in Ham's F12 medium, but with higher cell density in all the culture stages. Chondrocyte proliferation was maintained until passage 3 (P3) and the culture reached confluence at 2 weeks (Fig. 1F).

Human Nasal Septum Chondrocyte Growth Rate And Viability

Chondrocytes cultured in the F12:DMEM (1:1) medium achieved the highest growth rate (4.2×10^3 average increase in cell number per day/cm^2) in the primary culture (P0). And this was further confirmed by the statistical analysis that showed the growth rate in F12:DMEM was indeed significantly higher than in either of the individual media alone ($p<0.05$) at P0 (Fig. 2).

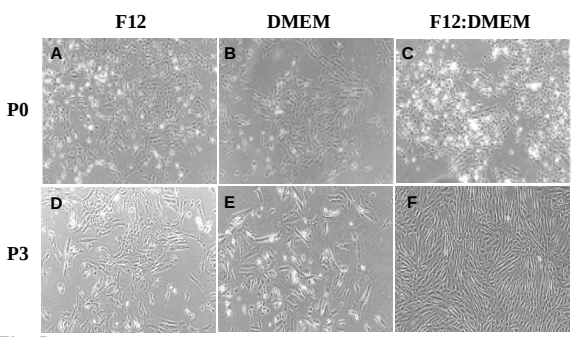


Fig 1. Human nasal septum chondrocyte primary culture (P0) at 2 weeks in Ham's F12 medium (F12) (**A**), Dulbecco's Modified Eagle Medium (DMEM) (**B**) and an equal volume mix of Ham's F12 medium and Dulbecco's Modified Eagle Medium (F12:DMEM) (**C**) compared to the passage 3 (P3) cultured chondrocytes at 2 weeks in Ham's F12 medium (**D**), Dulbecco's Modified Eagle Medium (**E**) and F12:DMEM (**F**). Cultured chondrocytes at P3 appeared more elongated in shape and bigger in size compared to the primary culture (40X).

As the cultured chondrocytes were passaged from P0 to P1, their growth rate decreased about 50% in all three types of media (Fig. 2a). The combination medium (F12:DMEM) continued to support a significantly higher growth rate ($p<0.05$) compared to F12 and DMEM individually.

Chondrocytes in passage 2 (P2) showed a further decrease in growth rate with chondrocytes cultured in F12:DMEM scored 1.8×10^3 cells/day/cm^2, followed by DMEM with 1×10^3 cells/day/cm^2 and lastly F12 with only 0.8×10^3 cells/day/cm^2 (Fig. 2a). Chondrocyte growth rate in the combination medium (F12:DMEM) was still significantly higher ($p<0.05$) compared to that for the chondrocytes cultured in F12 or DMEM.

At passage 3 (P3), chondrocytes cultured in the individual media; F12 or DMEM did not reach confluence even after a long culture period (3 weeks). Cell multiplication activity was hardly seen under the inverted light microscope. The sub-confluent culture was trypsinized after 21 days and total cells were counted. The combination medium (F12:DMEM) continued to promote chondrocytes proliferation and all the samples reached confluence by 2 to 3 weeks. As in previous passages, the chondrocyte growth rate in F12:DMEM remained significantly higher than both F12 and DMEM in P3 stage ($p<0.05$) (Fig. 2a). There was no correlation between chondrocyte growth rate and the age of donor in this study.

Chondrocytes cultured in the P0, P1, P2 and P3 stages showed more than 90% viability in all the three types of medium used. There was no significant difference for chondrocyte viability between these groups.

Human Nasal Septum Chondrocytes Population Doubling Time

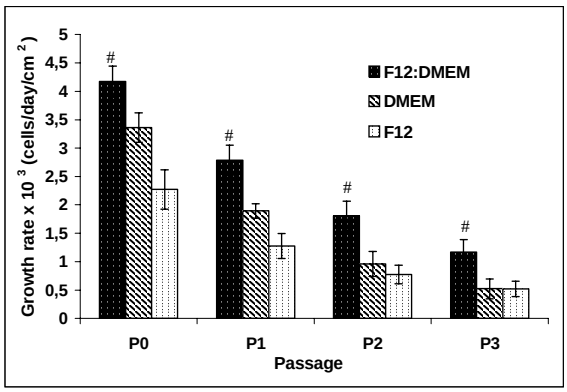
Chondrocytes cultured in the combination medium (F12:DMEM) showed significantly ($p<0.05$) shorter population doubling time (PDT, 3.0 days) compared to DMEM (3.6 days) and F12 (4.6 days) in primary culture (P0; Fig. 2b).

As shown in figure 2b, the chondrocytes' PDT increased as the cultures underwent passages. The degree of PDT increment was the greatest in DMEM from passage 1 (5.1 days) to passage 3 (29.7 days). The chondrocytes' PDT in F12 increased from 7.1 days at passage 1 to 14.9 days at passage 3. Chondrocytes cultured in the mixed medium (F12:DMEM) showed a moderate increment, from 3.7 days at passage 1 to 7.0 days at passage 3. At the culture stages of passage 1, passage 2 and passage 3, the chondrocytes' PDT in F12:DMEM was significantly shorter than F12 or DMEM. There was no correlation between

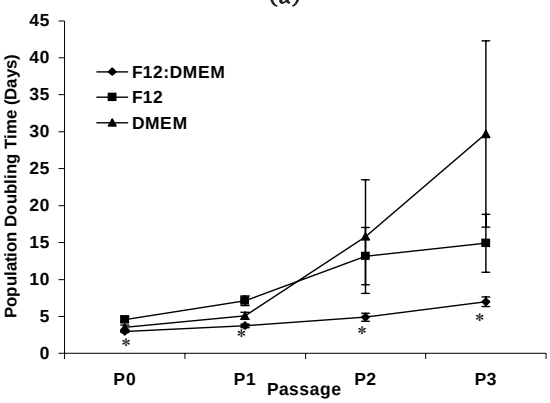
chondrocytes PDT with the age of donor in this study.

Human Nasal Septum Chondrocytes Total Number of Cell Doublings

The total number of cell doublings indicates the magnitude of chondrocytes expansion in culture. Scores for the number of cell doublings in every culture stage were accumulated to obtain the total number of cell doublings in the different culture media. Chondrocytes cultured in F12:DMEM (1:1) scored the highest total number of cell doublings (11.2 times) with an increase of 2.4×10^3 fold in cell number. Chondrocytes cultured in DMEM scored 10.1 total number of cell doublings with a 1.1×10^3 fold increase in cell numbers. Chondrocytes cultured in Ham's F12 medium only had 8.8 total number of cell doublings, which means a 0.4×10^3 fold increase in cell number. There was no correlation between chondrocytes total number of cell doubling with the age of donor in this study.



(a)



(b)

Fig 2.(a) Growth rate (average increase in cell number per day/cm^2) of human nasal septum chondrocytes cultured in HAM's F12 medium (F12), Dulbecco's Modified Eagle Medium (DMEM) and a equal volume mix of Ham's F12 medium and Dulbecco's Modified Eagle Medium (F12:DMEM) at primary culture (P0), passage 1 (P1), passage 2 (P2) and passage 3 (P3). All values were showed as mean ± SEM (n = 9). # Chondrocytes growth rate in F12:DMEM medium was significantly higher than both F12 and DMEM at all the passages ($p<0.05$).
(b) Population Doubling Time (PDT; days) of human nasal septum chondrocytes cultured in HAM's F12 medium (F12), Dulbecco's Modified Eagle Medium (DMEM) and a equal volume mix of Ham's F12 medium and Dulbecco's Modified Eagle Medium (F12:DMEM) at primary culture (P0), passage 1 (P1), passage 2 (P2) and passage 3 (P3). All values are shown as mean ± SEM (n=9). * PDT of cultured chondrocytes in F12:DMEM was significantly shorter than both F12 and DMEM at all the passage stages ($p<0.05$).

Gene Expression of Human Nasal Septum Chondrocytes Grown in Monolayer Culture

One step reverse transcriptase-polymerase chain reaction analysis of the freshly isolated chondrocytes (n=9) showed only type II collagen gene expression and no type I collagen gene expression (Fig 3a). Chondrocytes cultured either in Ham's F12 medium, Dulbecco's Modified Eagle Medium or the equal volume mix of Ham's F12 medium and Dulbecco's Modified Eagle Medium (F12:DMEM) from all samples (n=9) showed no difference on the gene expression pattern. All cultured chondrocytes from primary cultures (P0) actively expressed both type I and type II collagen genes (Fig 3a). Though the degree of type I collagen gene at P1 and P2 stages were maintained in all the three types of medium used, the expression level of type II collagen gene expression seemed to slowly diminish after the P1 stage and finally was not detectable in the subsequent passage (P2)(Fig 3a). Again, this gene expression pattern was the same regardless of the types of medium used to culture the chondrocytes (n=9). Expression of the β-actin gene was steadily maintained in all the cultures, thereby confirmed that the RT-PCR analysis was successful (Fig 3a).

Gene Expression Evaluation on the Isolated Cells from Engineered Tissue

Via one step reverse transcriptase-polymerase chain reaction, total RNA extracted from the cells of engineered tissue was used to analyze the expression of the type I and type II collagen genes (n=9). Surprisingly, cells isolated from the *in vivo* engineered tissue demonstrated re-expression of the type II collagen gene after losing this phenotype in the extensive culture expansion in F12:DMEM (Fig. 3b). However, the type I collagen gene remained co-expressed along with the type II collagen gene.

Histology Evaluation on Engineered Cartilage Tissue

Only the combination medium of F12:DMEM (1:1) provided sufficient numbers of chondrocytes for cartilage tissue engineering. As predicted, the engineered constructs made of pluronic F-127 scaffold alone (control) became smaller after implantation, and totally dissolved 2 weeks later. On the other hand, the engineered tissues made from pluronic F-127 scaffold mixed with cultured chondrocytes developed into solid tissues after

8 weeks of implantation. When these *in vivo* engineered tissues were removed, they appeared glistening white in color and firm in consistency. Hematoxylin & eosin staining on the paraffin sections demonstrated the engineered tissues consisted of evenly spaced lacunae cells embedded in a basophilic matrix. The lacunae cells were variable in shapes from round to oval with slight pleomorphism (Fig. 4a). Safranin O staining on the sectioned tissues was strongly positive, correlating with abundant proteoglycans production. A thin perimeter layer resembling perichondrium was also demonstrated on the engineered tissues (Fig 4b).

Discussion

In this study human nasal septum chondrocytes were successfully isolated from a broad range of age from 20 to 60 years old as also shown by Rotter et al. (2001)[8]. The yield of the freshly isolated chondrocytes in this study was (2.1 x 10^3 cells per mg of cartilage) and this was indeed higher compared to chondrocytes harvested from human rib cartilage (300 chondrocytes per mg)[9]. Furthermore, nasal septum chondrocytes were usually high in viability and able to proliferate well in the selected culture media regardless of the age of the donor.

As cells underwent serial passages, their morphology changed from polygonal to spindle-like shape, which had been called a dedifferentiation process by other groups [11, 22-24]. The elongation phenomenon was believed to be due to some modification in the cytoskeleton[22], and was most prominent in chondrocytes cultured in Dulbecco's Modified Eagle Medium (DMEM). Previous study showed that human meniscus cells proliferated faster in DMEM with an additional 10% FBS rather than in Ham's F12 or the equal mix medium (F12: DMEM)[23]. Morphologically, these cells became elongated faster in DMEM and further analysis on the molecular level revealed that the cells lost its native cartilage phenotype expression[23]. A later study by Baek et al. (2002) showed that the use of poly-HEMA coated dishes helped rat rib chondrocyte in culture to maintain their morphology, therefore maintaining better phenotypic expression than in normal monolayer culture[24].

Our study demonstrated that human nasal septum chondrocyte grew significantly faster and the growth rate was higher in the combination medium (F12:DMEM; 1:1 ratio) compared to the normally used individual basal medium. The chondrocytes number at every passage was noted to show an average increase as high as 10 to 15 fold in the F12:DMEM medium. The population doubling time for human nasal septum chondrocytes in the same medium was shorter, which means the culture took shorter time to reach confluence in F12:DMEM (1:1) than in the two other media used. A similar study done by Rotter et al. (2001)[8] reported that the doubling time of human nasal septum chondrocytes primary culture in DMEM was 2.6 days, which was slightly shorter than the doubling time of 3 days in chondrocytes cultured in F12:DMEM (this study). This difference might be due to higher seeding density practiced by Rotter et al. (2001)[8]; 8,000 cells/cm^2 compared to 5,000 cells/cm^2 (this study).

Human nasal septum chondrocytes scored the highest total number of cell doublings (11.2 cell doublings) in the combination medium (F12:DMEM). With this medium, the initial number of human nasal

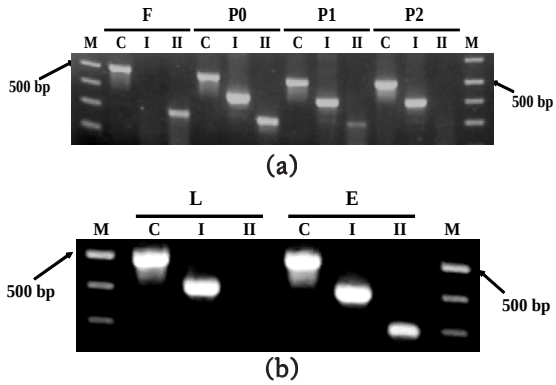


Fig 3. **(a)** One step reverse transcriptase-polymerase chain reaction analysis of the expression of type I and type II collagen genes from freshly isolated chondrocytes (**F**) and cultured chondrocytes at different culture stages: primary culture (**P0**), passage 1 (**P1**) and passage 2 (**P2**) (n=9). Freshly isolated chondrocytes only expressed type II collagen gene with no expression of type I collagen gene. Cultured chondrocytes expressed both type I and type II collagen genes in primary culture (P0). At passage 1 (P1), expression of the type II collagen gene was reduced whilst expression of the type I collagen gene remained active. Cultured chondrocytes at passage 2 (P2) showed no expression of the type II collagen gene, but the type I collagen gene was continually expressed. This gene expression pattern was the same regardless of the type of medium used to culture the chondrocytes. Expression on β-actin gene was chosen as a control. Lane M: 100 bp DNA marker. Lane C: β-actin gene fragment (495 bp). Lane I: Type I collagen gene fragment (396 bp). Lane II: Type II collagen gene fragment (294 bp).
(b) One step reverse transcriptase-polymerase chain reaction analysis of the expression of type I and type II collagen genes from the large scale expanded chondrocytes (**L**) and cells isolated from engineered tissue (**E**) (n=9). Large scale expanded chondrocytes in F12:DMEM expressed only the type I collagen gene with no expression of the type II collagen gene. Cells isolated from the engineered tissue demonstrated re-expression of the type II collagen gene. Expression of the β-actin gene was chosen as a control. M: 100 bp DNA marker. Lane C: β-actin gene fragment (495 bp). Lane I: Type I collagen gene fragment (396 bp). Lane II: Type II collagen gene fragment (294 bp).

septum chondrocytes was able to be expanded more than 2,000 times within 8 weeks. This result is important in the tissue engineering field because it confirmed that human nasal septum chondrocyte can be effectively culture-expanded in a short period of time without the addition of any growth factors.

Gene expression analysis on the collagen genes (type I and type II) showed that continual expansion of chondrocytes would cause down regulation of type II collagen gene expression and this phenomenon was noticeable in all the three types of media used in the study. This dedifferentiated phenotype expression was similar to that reported in two other studies with human nasal septum chondrocytes[24] and rabbit auricular chondrocytes[25]. Since the type of medium used was unable to maintain the differentiated phenotype of chondrocytes, modification of the culture system or growth factor supplementation will be considered in future studies.

In this study, we demonstrated the formation of engineered cartilage by using pluronic F127 mixed with cultured human nasal septum chondrocytes. Pluronic F127 was also reported to be a good biomaterial to be used with swine auricular chondrocytes[26,28] and human auricular chondrocytes[27]. At 4°C, this biomaterial is in a liquid state and can be mixed well with chondrocytes. It also served as a good scaffold material for cartilage tissue engineering as proven by the histological findings (Fig. 4). When warmed up to physiological temperature (37°C), it transformed into a thick semisolid gel and thus, provided a 3-dimensional environments that allowed chondrocytes to regain their roundness and later promoted matrix production. The dedifferentiation process that led to the absence of collagen type II expression in chondrocytes after prolong expansion seem to be reversible after 8 weeks of *in vivo* implantation, and collagen type II was re-expressed. The reversibility might be due to the 3-dimensional environment provided by the hydrogel, which later promotes the normal phenotype gene expression. This observation was also supported by other research findings; cultured human nasal septum chondrocytes eventually would regain their former chondrocytic characters after being grown in a 3-dimensional environment either on soft agar[28] or in alginate system[29]. The presence of type I collagen gene expression even in *in vivo* engineered tissues suggested the existence of a thin layer of membrane resembling perichondrium which covered the engineered cartilage (Fig. 3b). Due to the difficulty of separating the layer, total RNA extracted from this part of tissue might be responsible for the expression of the type I collagen gene detected in the analysis. Expression of the type I collagen gene may also be contributed by the presence of immature cells in the engineered tissue[30]. The Safranin O staining of the engineered construct demonstrated abundant proteoglycan production, which is comparable with native human nasal septum cartilage. These results showed that our engineered human cartilage has high potential for future clinical application.

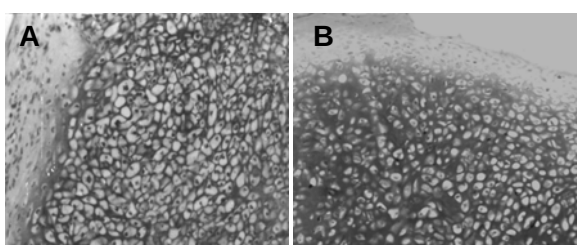


Fig 4. Histological evaluation of the engineered tissue removed from nude mice after 8 weeks of *in vivo* implantation. (**A**) Hematoxylin & eosin staining demonstrated the engineered tissue consisted of evenly spaced lacunae cells embedded in a basophilic matrix. The lacunae cells were varied in shape; from round to oval with slight pleomorphism. (**B**) Safranin O staining was strongly positive, correlating with abundant proteoglycans production. A thin perimeter layer resembling perichondrium was also demonstrated on the engineered tissue. No fibrous tissue or vascular ingrowths were noted (100X).

This study has proven that cartilage engineering may start from a limited biopsy tissue and this is relevant in clinical situations. The amount of cartilage obtained from patients in this study was small (≈ 200 mg), and the isolated chondrocytes were then divided equally for each medium (F12, DMEM, F12:DMEM), which resulted in 50,000 chondrocytes for initial seeding in primary cultures. Only F12:DMEM (1:1) was able to provide sufficient numbers of cultured chondrocytes to form engineered tissue. HAM's F12 and DMEM alone both failed to produce enough chondrocytes for the reconstruction of engineered cartilage. Though the present study highlighted the advantage of using the combination of F12:DMEM (1:1) medium in propagating human nasal septum chondrocytes *in vivo*, there are still some factors that remain to be studied, such as the effect of each component listed in each medium on cell expansion and phenotype characteristics.

Conclusion

The equal volume combination of Ham's F12 and Dulbecco's Modified Eagle Medium promoted a higher proliferation rate of human nasal septum chondrocytes, as evidenced by their shorter doubling time and higher growth rate. This information is of critical importance in the effort to translate tissue engineering into clinical practice. The combination medium supplemented with 10% FBS in culture may effectively expand the number of human nasal septum chondrocytes for cartilage tissue engineering purposes. Hydrogel Pluronic F-127 is suitable to be used as a biomaterial for human cartilage reconstruction as it provides a 3-dimensional environment

that maintains the phenotype of the chondrocytes.

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