TISSUE ENGINEERED CARTILAGE WITH DIFFERENT HUMAN CHONDROCYTE SOURCES: ARTICULAR, AURICULAR AND NASAL SEPTUM

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SUMMARY: Tissue engineering is a new biotechnological field that has emerged recently, providing an alternative to the treatment of damages and defects of human tissues. Amongst the most researched tissues is cartilage. In this study, the general characteristics of chondrocytes derived from three different sources in the body: articular, auricular and nasal septum cartilage, were investigated, looking both at cellular and tissue construct levels. With the approval of Research and Ethical Committee of Medical Faculty, Universiti Kebangsaan Malaysia, redundant cartilages from human articular, auricular and nasal septum were obtained from several patients during surgical procedures. Samples were digested and chondrocytes obtained were cultured in a mixture of Ham's F12 : DMEM medium. All three chondrocyte sources were found to behave quite similarly in terms of cell morphology, gene expressions and histological analysis. No significant difference of the growth rates was shown by the three chondrocyte sources. Histological analysis via H and E and Safranin O stainings of the tissue-engineered cartilages demonstrate resemblance to the native cartilage. Gene expression results showed that collagen type II expression reduces after every passage, and collagen type I gene is expressed as early as P0, however, there were re-expressions of Collagen Type II gene in the in vivo construct. We have successfully engineered cartilage tissues which are similar to the native cartilage, using sources from articular, auricular and nasal septum chondrocytes.

Key Words: Tissue engineering, articular, auricular, nasal septum cartilage, chondrocytes.

INTRODUCTION

Tissue engineering is a multidisciplinary field which seeks into the generation of new biological substitutes using cells as the main ingredient, to replace damaged and defective tissues. Cells are derived from a small piece of tissue specimen taken from either bone, skin, cartilage or any other parts of the human body, usually the same type of tissue to be regenerated. In this study, cartilage specimens from patients aged 21-52 years were used. The validity for the age range is based on a study, which reported that cartilage engineered from the cells of nasal septum specimens showed no significant dependence on the age of the donor (6). Thus, it was assumed that the same justification applies to auricular and articular chondrocytes.

For clinical application, a limited amount of autologous cartilage specimen should be expected in order not to induce scar and morbidity at the donor site. Thus, only

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limited number of chondrocyte can be isolated from the specimen. However, these chondrocytes can be multiplied abundantly by cell culture technique in order to get enough cells, to generate a new tissue. Although it takes some time, a potentially limitless supply of functional tissue could be engineered by expanding cells from a small amount of donor tissue *in vitro* (1). Therefore, medical scientists foresee this fact as a beneficiary outcome for a limitless usage that promises a high technological quality of medical treatment.

It was reported that constructs made with auricular chondrocytes had the highest modulus and glycosaminoglycan content and the lowest permeability value and water content, while constructs made with articular chondrocytes had the lowest modulus and glycosaminoglycan content and the highest permeability value and water content (1). The reasons behind these could be related to the cellular basis or even more specific, the molecular contents of the cells.

The objectives of this study were to compare the growth rate, morphology, gene expressions of the chondrocytes and the observation of the structures of the tissue-engineered constructs via histological analysis. This includes all chondrocytes derived from different sources which are articular, auricular and nasal septum, in the attempts to engineer human cartilage with superior quality depending on their various uses in medical treatment.

MATERIALS AND METHODS

Chondrocytes isolation from cartilage

Excessive cartilage specimens were obtained after consent from 18 patients with the age ranging from 21 to 52 years after various corrective surgeries. These specimens would usually be discarded. Their uses in this study have been approved by the Ethical Committee of Medical Faculty, Universiti Kebangsaan Malaysia. Each piece of cartilage was separated free from the perichondrium, minced into small pieces ($1mm^3$), washed with phosphate buffer saline (PBS; pH7.2; Gibco, Grand Island, NY) containing 100U/mL of penicillin (Gibco), 100 mg/mL of streptomycin (Gibco) and 0.25 mg/mL of amphotericin B (Gibco) and was digested with 0.6% collagenase II (Gibco) at 37°C for 12 hours. After digestion, the suspension containing isolated chondrocytes was centrifuged at 6,000 rpm for 5 minutes. The resulting cell pellet was washed twice with PBS to remove remaining digestive enzyme before re-suspending in PBS for total cell quantification with a haemacytometer (Weber Scientific International Ltd. Middlx, England) and cell viability determination with trypan blue vital dye (Gibco).

Chondrocytes culture expansion

Isolated chondrocytes were seeded in six-well tissue culture plates (Falcon, Franklin Lakes, NJ) as the primary culture (P0) with a density of 5,000 cells/cm^2 in an equal volume mixture of Ham's F12 medium and Dulbecco's Modified Eagle Medium (F:D; Gibco). The media was supplemented with 10% fetal bovine serum (FBS; Gibco). Other supplements in the medium were 200mM L-glutamine, 100U/mL of penicillin, 100mg/mL of streptomycin, 0.25 mg/mL of amphotericin B (Gibco) and 50 mg/mL of ascorbic acid (Sigma, St. Louis. MO). All cultures were maintained in 5% CO_2 incubator (Jouan, Duguay Trouin, SH) at 37°C with culture medium changed two to three times a week. Cell cultures were examined everyday by inverted light microscope (Olympus, Shinjuku-ku, Tokyo). When the primary cultures (P0) reached confluence, they were trypsinized with 0.05% trypsin-EDTA (Gibco) and the harvested chondrocytes were washed and re-suspended in PBS for total cell number determination and cell viability test. Then, cultured chondrocytes were passaged three times (P1, P2 and P3) with the same cell density and growth environment. Chondrocytes growth rate (cells/day/cm^2) in every culture stage was calculated for statistical analysis. The data were expressed as mean ± standard error of the mean (SEM).

Large-scale chondrocytes culture and cartilage reconstruction

Chondrocytes were plated in 175 cm^2 culture flasks (Falcon, Franklin Lakes, NJ) at a density of 5000 cells/cm^2. Confluenced cells were then harvested by trypsinization with 0.05% trypsin EDTA (Gibco). The culture-expanded chondrocytes were suspended in human fibrin and polymerized with 1M calcium chloride solution for the formation of engineered cartilage. The engineered cartilages were kept *in vitro* for three weeks before being implanted (*in vivo*) subcutaneously in the nude mice.

Engineered cartilage evaluation by histological analysis

Engineered cartilages were removed from nude mice after 8 weeks of *in vivo* implantation. The engineered cartilages (*in vivo* constructs as well as the *in vitro* constructs) were divided into two portions for two different processes for the evaluation purposes. One portion of the engineered cartilage was fixed with 10% formalin for 24 hours and then processed for paraffin embedding. The paraffin blocks were then sectioned and serial sections were stained with Hematoxylin and Eosin (H and E) to evaluate the cell morphology and tissue organization. Safranin O staining on the tissue sections was to assess cartilage quality of the tissue, where by orange-red staining indicate cartilage proteoglycans production in the tissue. Verhoeff's Van Gieson staining on the tissue sections was to detect the elastin fiber which is indicated by dark blue staining.

For immunohistochemical analysis on type II collagen production, slide sections were incubated with proteinase K (Sigma) at 37°C for 45 minutes. Mouse antihuman type II collagen antibody (sigma) at concentration of 1:100 was then applied on the section for 30 minutes followed by anti-mouse streptavidin-perox-

idase complex (Dako, Glostrup, Denmark) for 15 minutes and color development with DAB substrate (Dako) as chromogen.

Another portion was digested using collagenase II for gene expression evaluation, using the same techniques as applied to digest cartilage specimens.

Gene Expression Analysis by One Step Reverse Transcriptase-Polymerase Chain Reaction

Total RNA from cultured chondrocytes at various culture stages, engineered tissues and native cartilages were extracted using TRI Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's instruction. Polyacryl Carrier (Molecular Research Center) was added in each extraction to precipitate the total RNA. Extracted RNA pellet was then washed with 75% ethanol and dried before dissolved in RNAse and DNAse free distilled water (Invitrogen, Carlsbad, CA). Yield and purity of the extracted RNA was determined by spectrophotometer (Bio-Rad, Hercules, CA). Total RNA was stored at -80°C immediately after extraction.

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 ß-actin gene was used as control. The specific sense and antisense primers used in the reaction were designed from listed NIH GenBank database and had the following sequences: type I collagen, 5'-AAGGCTTCAAGGTCCCCCTGGTG-3' and 5'-CAG CACCA-GTAGCACCATCATTTC-3'; type II collagen, 5'-CTG-GCAAAGAT GGTGAGACAGGTG-3' and 5'GACCATCAGT-GCCAGGAG TGC-3'; β-actin, 5'-CCGGCTTCGCGGGCGACG-3' and 5'TCCCGGCC AGCCAGGTCC-3'. One Step RT-PCR reaction mix was prepared according to the manufacturer's instruction with slight modification of using 25 mL volume reaction instead of 50 mL volume reaction. This modification was made to increase the sensitivity of the test. Each reaction consisted of 100 ng total RNA and 10 pmol of each sense and antisense primers. One step RT-PCR was performed in a 9700 thermal cycler (Perkin Elmer, Norwalk, CT) with reaction 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 sec at 94°C, 30 sec at 60°C and 30 sec at 72°C. This series of cycles was followed by a final extension of 72°C for 2 min. Subsequently, the PCR products were separated by electrophoresis 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).

Statistical analysis

Data of chondrocytes total cell yield, time upon reaching confluency and viability in each medium at every passage (P0, P1, P2 and P3) were collected from all samples. Values are presented as mean ± standard error of mean (SEM). Student's t-test was used to compare the data in each of the groups. Differences at the 5% level were considered significant.

Figure 1: All figures show the chondrocytes monolayer at P0 and after serial passages with magnification X40. Left-hand column: Articular chondrocytes, Middle column: Auricular chondrocytes and Right-hand column: Nasal septum chondrocytes. General morphology of chondrocytes at P0 is polygonal shape and after serial passages spindle shaped. There are no obvious morphological differences among the three types of cell sources.

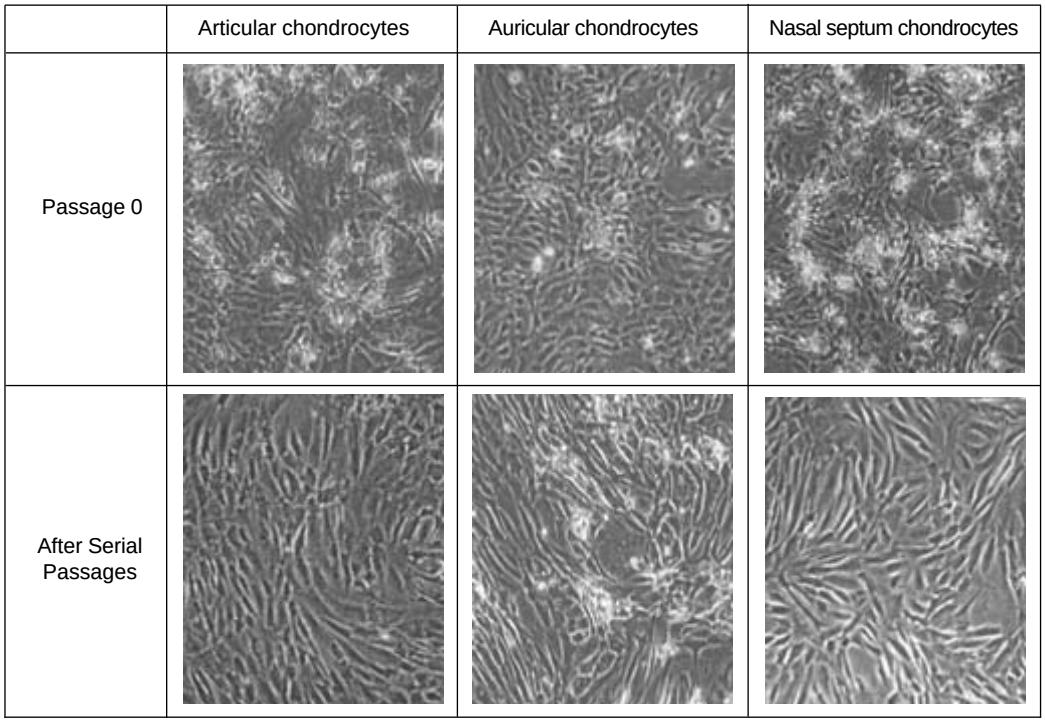
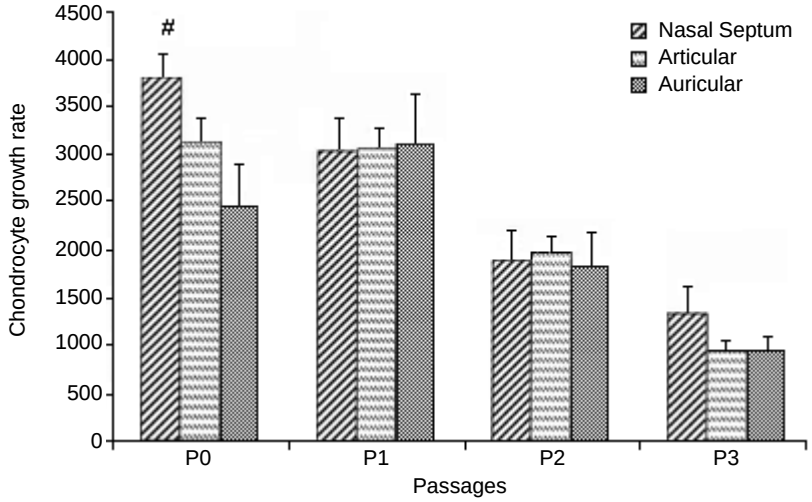


Figure 2 : The graph shows the growth rate of three types of chondrocyte at P0-P3, grown in FD. T-test result shows no significant difference between the types of chondrocyte of each passage except for nasal septum chondrocyte at P0 which is significantly greater than auricular chondrocyte at P0. Form the t-test carried out, P = 0.021 assuming equal variances.



RESULTS

Morphologic features of monolayer chondrocytes

The features of chondrocytes from articular, auricular and nasal septum, both at PO and after serial passages respectively were similar in each level (Figure 1). For all three of the chondrocyte sources at P0, the cells resemble polygonal morphology, however the cells switched into spindle morphology after serial passages. There was no obvious morphological difference among the three types of cell sources both at PO and after serial passages, which means that all types were behaving similarly.

Growth rate

The growth rate of articular and nasal septum chondrocytes grown in FD media decreases from P0 to P3 (Figure 2). A different trend was observed for auricular chondrocytes which showed an increase in the growth rate from P0 to P1, but decreased after that stage onwards. Nasal septum showed the best proliferation rate at P0, significantly higher than auricular chondrocytes. Student's t-test showed no significant difference between the three samples at each passage respectively.

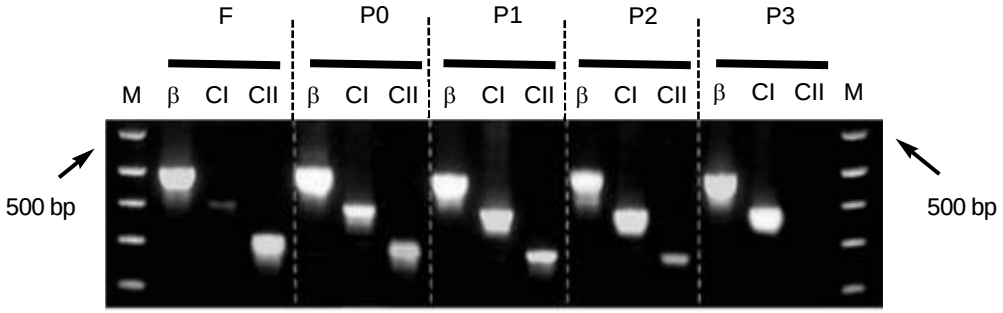
Gene expression

One step reverse transcriptase-polymerase chain reaction (One Step RT-PCR) demonstrated the gene expressions of auricular chondrocytes monolayer at fresh digest, PO, P1, P2 and P3 (Figure 3a) and the native cartilage, *in vitro* and *in vivo* constructs made with auricular chondrocytes (Figure 3b). For all three chondrocytes sources, similar cartilage gene expressions pattern were displayed for every passage. Collagen type II gene is

Table 1: Data used for graph construction (Figure 2) from the t-test carried out, p = 0.021 if equal variance assumed. Paper should be categorized under Tissue Engineering.

	Nasal Septum		Articular		Auricular	
Passage	Growth Rate/day	SEM	Growth Rate/day	SEM	Growth Rate/day	SEM
PO	3809.5	240.8905	3138.667	241.8492	2469.333	427.9806
P1	3041.167	348.6327	3061.333	217.9418	3117.667	506.2254
P2	1896.167	307.539	1999.5	159.1037	1852.667	339.7819
P3	1355.833	307.8422	967	108.3919	962.3333	165.1447

Figure 3a : This diagram shows the changes in expression of genes in human auricular chondrocytes in monolayer, with increasing passage in culture, detected using one-step RTPCR with β actin gene as the control. Freshly isolated chondrocytes (F) expressed type II collagen strongly and very weak expression of type I collagen, P0 and P1 culture expressed both types I and II collagens, at P2 type II collagen expression began to reduce and finally at P3, type II collagen showed negative expression. Similar gene expression pattern was displayed by the articular and nasal septum chondrocytes.



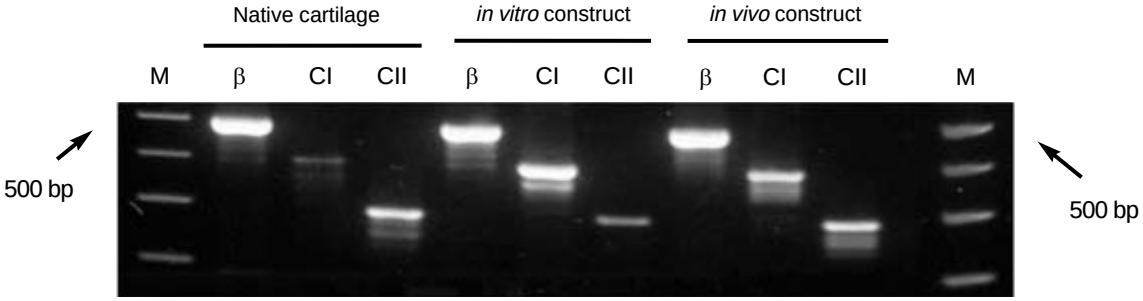
M: DNA marker, β: β actins gene, CI: collagen I, CII: collagen II.

expressed by monolayer culture of articular, auricular and nasal septum during PO and PI, and a very minimal expression during P2. At P3, collagen type II gene is not expressed at all. Collagen type I gene was not expressed by cells after fresh digestion. However, after culture as early as PO, collagen type I gene was expressed, and the same effect occurred throughout P1, P2 and P3. Collagen gene type II was expressed in all native cartilage, *in vitro* and *in vivo* constructs.

Histological Analysis

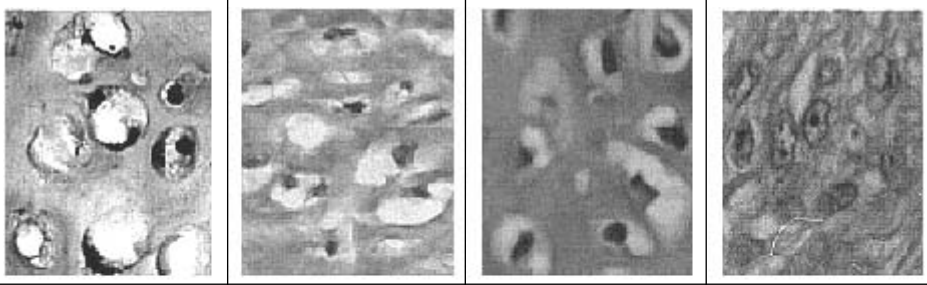
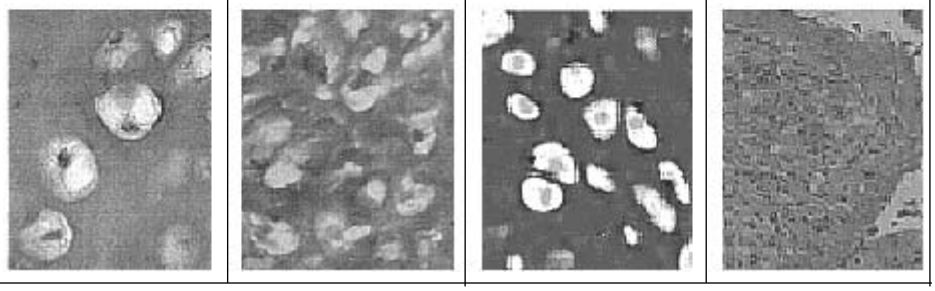
Micrographs of histological stainings of the *in vitro* and *in vivo* constructs are shown in Figures 4a and 4b. Haematoxylin and Eosin (H and E) and Safranin-O staining of the *in-vivo* constructs of all three samples all show positive results, which resembles that of the native cartilage. From the H and E stain, the *in vivo* constructs of the engineered tissues demonstrated the same morphological characteristics as the native cartilage, with the cells located within typical chondrocyte lacunae and surrounded by cartilaginous matrix. Meanwhile, positive Safranin-O stain indicates the presence of sulfated proteoglycans. In contrast, the *in vitro* constructs are all negatively stained and show immature chondrocytes. Verhoeff's Van Gieson staining of the *in vivo* constructs did not show positive results, which mean that elastic fibers are not present in the samples. Immunohistochemical stain using collagen types II and I of the *in vivo* constructs of all three types of the chondrocytes showed positive staining in Figure 4b, whilst the *in vitro* constructs all showed negative staining. This shows that collagen type II and collagen type I genes were only present after *in vivo* implantation.

Figure 3b : This diagram shows type I and II collagens gene expression *in vitro* and *in vivo* constructs of human auricular chondrocytes compared to the native cartilage by using the one-step RTPCR with β actins gene as the control. Native cartilage expressed type II collagen strongly and very weak expression of type I collagen, whereas the constructs expressed type I and II collagens. Similar pattern of gene expressions were noted for the other two sources of chondrocytes.



M: DNA marker, β: β actins gene, CI: collagen I, CII: collagen II.

Figure 4a : Histological analysis: Micrographs of Hematoxylin and Eosin and Safranin O staining of tissue-engineered constructs are tabulated below. *In vitro* state features immature chondrocytes which is different from *in vivo*, which shows matured chondrocytes lying in lacunae, similar to native cartilage. Positive Safranin O stainings of *in vivo* constructs indicates the presence of abundant proteoglygan in contrast to the *in vitro* constructs which shows negative staining. The photomicrographs of engineered cartilage made only from articular chondrocytes are shown for *in vitro* constructs because all look similar.

	In vivo			*In vitro*
	Articular	Auricular	Nasal Septum	
Hematoxylin and Eosin				
Safranin O				

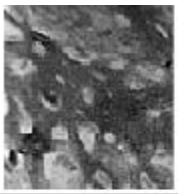
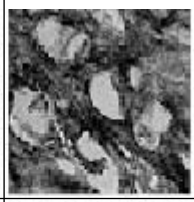
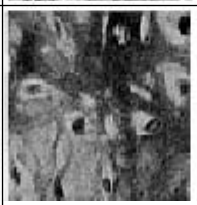
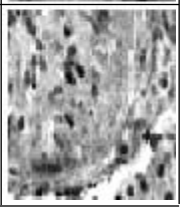
DISCUSSIONS

It was reported earlier that cultured nasal septum chondrocytes had polygonal morphology at PO and switched to spindle shape at P3 (13), while auricular chondrocytes morphology changed from polygonal in shape to be more elongated and bigger in size with passage 16. This observation is similar to the findings in this study for all chondrocyte sources used. One logical reason for the fact that chondrocytes switch from polygonal morphology to spindle morphology after serial passages is due to the nature of the two dimensional culture conditions which expose the cells to the gravitational pull, resulting in cells to lay flat in order to stabilize themselves. Other internal mechanisms might be occurring such as the change in phenotypic expressions of the chondrocytes from expressing collagen gene type II to expressing collagen gene type I (2).

Generally, the number of cells available is not sufficient after P0 expansion due to limited amount of donor tissue, and need to be further expanded in consecutive passages. However, culturing chondrocytes after consecutive passages causes a reduction in the growth rate (Figure 2) and hence takes more time to reach the number required besides sacrificing the quality of chondrocytes obtained. The proliferation rate of chondrocytes decreased with increasing age of donors even with the addition of growth factors (12). In this study, cells from the donor up to 52 years of age were used which may have affected the results obtained. Besides that, certain methodology needs to be adopted in culturing chondrocytes such as the addition of certain supplements to ensure proper maintenance of cells phenotypic expression.

The proliferation of articular chondrocytes and the synthesis of the extracellular matrix have often been hypothesized to require a specific set of conditions for optimal growth (4). First, as an avascular structure, articular cartilage is dependent on the set of requirements provided by synovial fluid within the joint space. Specifically, constant motion of synovial fluid over joint surfaces is vital for both the removal of waste and the delivery of nutrients, in addition to other factors present within the milieu. Second, the physical properties of articulating surface add

Figure 4b: Histological analysis: Immunohistochemical analysis of Articular, Auricular and Nasal Septum Constructs. All *in vivo* constructs showed positive staining for both collagen types I and II. All *in vitro* constructs revealed negative staining both for collagen types I and II. Engineered cartilage has to undergo a form of maturation process to turn into a matured and functional cartilage.

	In vivo			*In vitro*		
	Articular	Auricular	Nasal Septum	Articular	Auricular	Nasal Septum
Immunohisto-chemical analysis: Collagen type II						
Immunohisto-chemical analysis: Collagen type I						

a unique mechanical component to proper growth. Joints frequently transmit considerable pressure loads- forces that seem to be necessary for the optimal proliferation and differentiation of articular cartilage. In comparison, ear and auricular cartilage do not require the specific set of conditions essential for proper articular cartilage growth. The subcutaneous location of native ear cartilage and the relatively greater vascular supply ensures a closer approximation of growth conditions in the setting of craniofacial and musculoskeletal defects occurring outside of joint space 4. It should be expected that the same phenomenon occurs for the cells derived from each source to behave accordingly, in condition that the cells still maintain its original molecular properties. In support of this statement, it was reported that engineered cartilage from human nasal cells survived and grew during six weeks of implantation *in vivo* whereas articular cartilage constructs failed to survive (3).

In this study, collagen type I was expressed by the tissue engineered cartilage, which is not expressed by native tissue. It was said by Sasano *et al.* (10) that collagen type I is expressed in immature cartilage (10,11), which is acceptable in this study since the tissue engineered chondrocytes is a newly generated cartilage, and is an immature tissue. In this study, the *in vivo* construct were maintained only for 8 weeks, and it may take longer for the cartilage to reach maturity. It was shown that collagen type I was expressed steadily throughout the *in vitro* passage by tissue-engineered auricular chondrocytes (15) through RT-PCR analysis study, as well as by tissue-engineered articular chondrocytes through immunohistochemical stains against collagen type I (17).

From the histological analysis, differences are shown by the *in vitro* and *in vivo* constructs. In a static *in vitro* environment, there is a minimal interaction occurring to the chondrocytes besides being pooled together closely in the three dimensional construct form. With no function to perform, minimal surrounding forces exertion and relying solely on the diffusion of nutrients from the surrounding FD media to supply the basic nutrients needs. For the *in vivo* construct, integration with the body system guides the chondrocytes to the correct pathway to adapt, which induces the maturity process and making it a better system. Safranin O stains carried out on articular neocartilage (18) and nasal septum neocartilage (14), both indicated the presence of accumulated proteoglygan. This finding is in line with the finding in this study which revealed that all chondrocytes sources used showed the same phenomenon.

Chondrocytes adopt a fibroblast-like morphology in monolayer culture, which is accompanied by an increase in proliferation and an altered phenotype (9). However, we think that this may be occurring at a certain degree that has yet to affect the properties of the original chondrocytes as in its source, which is why the constructs were stained positive for Safranin O.

CONCLUSION

We have successfully engineered cartilage which has similar properties to the native cartilage from three sources namely articular, auricular and nasal septum chondrocytes. Slight differences of the properties are reasoned out to be the properties of immature cartilage, which will mature with time naturally in the body.

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