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The formation of human auricular cartilage from microtic tissue: An in vivo study

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ABSTRACT

Objectives: This study aimed to isolate, culture-expand and characterize the chondrocytes isolated from microtic cartilage and evaluate its potential as a cell source for ear cartilage reconstruction. Specific attention was to construct the auricular cartilage tissue by using fibrin as scaffold.
Study design: Cell culture experiment with the use of microtic chondrocytes.
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Methods: After ear reconstructive surgery at the Universiti Kebangsaan Malaysia Medical Center, chondrocytes were isolated from microtic cartilage. Chondrocytes isolated from the tissue were cultured expanded until passage 4 (P4). Upon confluency at P4, chondrocytes were harvested and tissue engineered constructs were made with human plasma polymerized to fibrin. Constructs formed later is implanted at the dorsal part of nude mice for 8 weeks, followed by post-implantation evaluation with histology staining (Hematoxylin and Eosin (H&E) and Safranin O), immunohistochemistry and RT-PCR for chondrogenic associated genes expression level.
Results: Under gross assessment, the construct after 8 weeks of implantation showed similar physical characteristics that of cartilage. Histological staining showed abundant lacunae cells embedded in extracellular matrix similar to that of native cartilage. Safranin O staining showed positive staining which indicates the presence of proteoglycan-rich matrix. Immunohistochemistry analysis showed the strong positive staining for collagen type II, the specific collagen type in the cartilage. Gene expression quantification showed no significant differences in the expression of chondrogenic gene used which is collagen type I, collagen type II, aggrecan core protein (ACP), elastin and sox9 genes when compared to construct formed from normal auricular tissue.
Conclusion: Chondrocytes isolated from microtia cartilage has the potential to be used as an alternative cell source for external ear reconstruction in future clinical application.
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1. Introduction

Microtia refers to the malformation of the external ear. It is characterized by a small underdeveloped pinna, abnormally shape or a total loss of its structure [1]. This condition occurs unilaterally and bilaterally, but commonly it is unilateral which affect 90% of the patient. This abnormality usually involves the malformation of the external ear canal. Patient with microtia can undergo reconstructive surgeries improve the physical look of the pinna for cosmetic purposes. Majority of microtia repair involved the use of rib cartilage from the patient. This requires the patient to undergone 3–4 surgical procedures for total reconstruction. Hence, reconstructive surgery for the treatment of congenital microtia still remains as one of the most complex procedures, especially on determining the donor site. The needs to find other sources for tissue reconstruction have always become the main focus of many scientists for many years. Extrusion of medpor, a synthetic material has always been a problem in ear reconstruction as the material used are hard and does not have the elastic properties of the ear cartilage. Some improvement with recent study showed

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extrusion can be reduced if the rib cage cartilage graft or polymer-based ear scaffolds are enveloped under temporoparietal fascia flap (TPF) [2,3]. Thus, the need to find other option for the treatment and development of ear reconstruction is of vital importance. As microtia reconstruction surgery is performed, the malformed ear cartilage is usually discarded as they are considered abnormally formed cartilage. There have not been many studies done on microtic cartilage. Some studies have been performed but unfortunately none or little has taken an interest to look into the cell properties and characteristics of chondrocytes derived from microtic sources [4,5]. Thus, there are little references that have been documented which showed the potential of these cells at its cellular level. An in vitro study on cell development and growth in monolayer culture is very important because it can give an overall view on the characteristics of the cells and this eventually can give scientist an understanding on how the cells behave [6]. Our previous study (Ishak et al. [7]) have characterized chondrocytes derived from microtic cartilage and these chondrocytes are the possible cell source for ear reconstruction even though it may be lacking stem cell properties. Thus this study is a continuation to evaluate whether chondrocytes isolated from microtic cartilage can be use as a possible cell source in ear reconstruction. In external ear reconstruction, the selection of a scaffold material is crucial as it poses challenges to maintain the 3D structure of the ear. In recent years, studies related to tissue engineering have been on the rise and it has given researchers and clinicians an alternative in treating patients. Research on tissue engineering with specific tissue of interest, for example articular cartilage [8,9], auricular cartilage [10,11], cochlear [12], trachea [13], cornea [14], skin [15] and nerve [16,17] have shown promising results which indicates high potential of translation. The use of fibrin as scaffold has been documented before [9,18,23,25,26,28]. Fibrin is a good autologous biomaterial as temporary scaffold as it can be isolated from patient's blood. Its characteristic of high biodegradability and autologous is importance in reducing rejection. The use of fibrin was based on the idea that it is a natural biomaterial involved in tissue healing. Fibrin may also helps in the healing process due to its gel-like nature which provides a homogenous distribution of cells within the construct [18,19]. Thus, in this study, we evaluated the ability of chondrocytes isolated and expanded from microtic cartilage as a cell source for ear reconstruction with the use of fibrin as a carrier and scaffold.

2. Methods

2.1. Isolation and expansion of human auricular chondrocytes

Chondrocytes were isolated from 6 consented patients who undergone ear surgeries namely meatoplasty and canalplasty. The cartilages that were harvested are part of concha cartilage. The removal of the concha cartilage has been part of the procedures mentioned earlier (meatoplasty and canalplasty). The tissue is processed and cultured as describe previously [7]. Briefly, harvested auricular cartilage (1–3 cm^3) was washed and minced by using PBS containing antibiotics and later digested using 10 ml 0.6% Collagenase Type II. Cell suspension was then centrifuged and the resulting pellet was washed three times with 10 ml PBS. Cells were calculated using haemocytometer and total cell viability was determined by trypan blue dye exclusion method. Fresh isolated cells were collected and then cultured in a 6 well-plates with the initial seeding of 5000 cells/cm^2. The culture were passaged four times and maintained in 5% CO_2 incubator at 37 °C with the culture media changed once in three days until enough number of cells is achieved (human ethics approval: 02-01-02-SF0444).

2.2. Formation of construct and implantation

Constructs were formed by mixing chondrocytes with plasma. The plasma was obtained from consented individuals. Basically, about 10 ml of blood is collected and later centrifuged at 5000 rpm for 5 min. Generally, chondrocytes at passage 4 were trypsinized with EDTA 0.05%. Later, the culture chondrocytes were centrifuge at 5000 rpm for 5 min to formed cell pellet. The amount of cell used for construct formation is 30×10^6 cell/ml plasma. Cells pellet was suspended in the plasma homogenously and 25 μl of calcium chloride ($CaCl_2$) (1 molar/ml plasma) was added to initiate the construct formation by fibrin polymerization. Immediately, the cell-fibrin suspension is poured into a 6-well plate to form a disk-shaped construct. Construct formed will be incubated for 24 h in 5% CO_2 incubator prior implantation. The chondrocytes-fibrin constructs were implanted subcutanenously at the dorsal part of the nude mice under general anesthesia of ketamine (50 mg/ml), xylazil (20 mg/ml), and zolatil (20 mg/ml). Care of the nude mice was carried out under the animal facility of the animal unit, Universiti Kebangsaan Malaysia (animal ethics approval: ENT/2007/GOH/14-NOV/207-NOV-2007-DEC-2009).

2.3. Post implantation analysis

After 8 weeks of implantation, the construct is extracted and analyzed with gross analysis, histological staining (Hematoxylin and Eosin (H&E) and Safranin O), immunohistochemistry analyses by using collagen type i and ii antibodies and real time-reverse transcriptase polymerase chain Reaction (RT-PCR) for chondrogenic associated genes expression.

2.3.1. Quantitative gene expression by real-time PCR

Total RNA of engineered cartilage was extracted according to TRI reagent protocol (Molecular Research Center, Cincinnati, OH) as describe previously [7]. Briefly, total RNA of cells at all passages was extracted using TRI reagent (Molecular Research Center, Cincinnati, OH). This is to evaluate any changes in the gene expression during cell expansion. Total RNA was extracted according to the manufacturer's instructions. Auricular chondrocytes differentiation (type II collagen, elastin and aggrecan core protein), dedifferentiation (type I collagen), hypertrophy (type X collagen) and human transcription factor (sox9) gene expression profile were quantified. Primers for human GAPDH, type I, II, X collagen, elastin, aggrecan core protein, and sox9 were designed with Primer 3 software based on the GeneBank database sequences corresponding to the specific gene accession number as stated in Table 1. The reaction kinetic of each primer set was verified with

Table 1
Primer sequence used in real-time PCR for quantitative gene expression analysis.

Gene	Accession no.	5′–3′	PCR product size
GAPDH	NM_002046	F: tcc ctg agc tga acg gga ag R: gga gga gtg ggt gtc gct gt	217
Type I collagen	NM_000088	F: agg gct cca acg aga tcg aga R: tac agg aag cag aca ggg cca	222
Type II collagen	NM_001844	F: cta tct gga cga agc agc tgg ca R: atg ggt gca atg tca atg atg g	209
Elastin	NM_000501	F: ggc ctg gag gca aac ctc tt R: cca cca act cct ggg aca cc	189
Aggrecan core protein	NM_001135	F: cac tgt tac cgc cac ttc cc R: acc agc gga agt ccc ctt cg	183
Sox9	NM_000346	F: gcg gag gaa gtg ggt gaa ga R: ccc tc cgc ttc agg tca gc	236

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