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TISSUE-ENGINEERED HUMAN ARTICULAR CARTILAGE DEMONSTRATES INTENSE IMMUNOPOSITIVITY FOR COLLAGEN TYPE II

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Abstrak: Kejuruteraan tisu adalah satu bidang bioperubatan yang menggabungkan pelbagai disiplin ilmu dengan mengaplikasikan prinsip serta kaedah kejuruteraan dan sains hayat untuk menghasilkan bahan gantian biologi yang berupaya dalam pengambilalihan semula, penstabilan serta pembaikan fungsi tisu. Makmal kami telah berjaya mengkultur kondrosit artikular manusia dan membentuk semula tisu rawan melalui kaedah kejuruteraan tisu yang dilihat sebagai berpotensi dalam terapi penggantian tisu rosak. Namun, kita masih perlu menganalisis sama ada tisu rawan yang dihasilkan melalui kaedah kejuruteraan tisu mempunyai persamaan kualiti dengan tisu rawan normal. Kajian ini dilakukan untuk menilai kualiti tisu rawan yang dihasilkan melalui kaedah kejuruteraan tisu dengan menggunakan teknik pewarnaan imunohistokimia (IHC). Tisu rawan artikular manusia diperoleh daripada tisu berlebihan yang diperoleh selepas prosedur pembedahan sendi. Sel kondrosit yang dipencilkan secara penghadaman enzimatik daripada sampel tisu rawan dikultur di dalam media kultur selama beberapa pasaj untuk mendapatkan jumlah sel yang mencukupi. Sel kondrosit tersebut kemudiannya ditransformasi kepada struktur atau konstruk tiga dimensi dengan menggunakan biobahan yang terbiodegradasi. Konstruk *in vitro* yang terhasil kemudiannya diimplan secara subkutanus pada tikus atimik. Selepas 8 minggu, konstruk tadi akan dikeluarkan daripada tikus atimik dan dianalisis menggunakan teknik pewarnaan IHC untuk mengenalpasti kehadiran kolagen jenis I, kolagen jenis II serta antigen protein permukaan fibrosit. Keputusan menunjukkan bahawa konstruk *in vitro* yang stabil berupaya membentuk tisu rawan artikular yang matang selepas implantasi dan menyerupai tisu rawan hialin normal. Konstruk *in vitro* juga menunjukkan pola ekspresi kolagen jenis I, kolagen jenis II serta antigen protein permukaan fibrosit yang jelas pada kawasan periselular. Manakala konstruk *in vivo* pula, menunjukkan sebaran ekspresi kolagen jenis II sebagai bukti terhasilnya tisu rawan hialin yang berkualiti. Sebagai kesimpulan, kami telah berjaya menghasilkan semula tisu rawan artikular manusia yang menyerupai tisu rawan normal dengan teknik pewarnaan IHC.

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Abstract: Tissue engineering is known as an interdisciplinary biomedical field that applies the principles and method of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function. Our laboratory has successfully cultured human articular chondrocytes and formed tissue-engineered cartilage, which is reliable for replacement of damaged tissue. Yet, we still need to analyse whether the tissue-engineered cartilage has similarity to the native cartilage. This study was designed to evaluate the quality of human articular cartilage using immunohistochemistry (IHC) technique. Human articular cartilages were obtained from excessive tissues from consented patients after joint surgery. Chondrocytes were isolated from tissue via enzymatic digestion and cultured in growth media for several passages to obtain sufficient cells. The cells were then transformed into three-dimensional *in vitro* constructs using biodegradable scaffold and were placed subcutaneously in the nude mice. Constructs were harvested after 8 weeks and were analysed by IHC technique to detect the presence of collagen type I, collagen type II and fibrocytes surface protein. The results showed that stable *in vitro* construct mature into tissue-engineered cartilage after implantation, which grossly resembled native cartilage. *In vitro* construct strongly expressed collagen type I, collagen type II and the fibrocytes surface protein at the pericellular. *In vivo* construct showed the distribution of collagen type II expression throughout the tissue, which denotes good quality hyaline cartilage. In conclusion, we have successfully engineered human articular cartilage which resembles the native tissue by IHC evaluation technique.

Keywords: Tissue Engineering, Human Articular Cartilage, Collagen Type II

INTRODUCTION

Articular chondrocytes are specialised cells of mesenchymal origin found exclusively in cartilage. Articular cartilage is an avascular tissue in which physical properties depend on the extracellular matrix (ECM) produced by chondrocytes. The components of the ECM are proteoglycans which is embedded within a framework of collagen fibrils consisting predominantly of collagen type II; water and other proteins and glycoprotein present in lower amount. These components combine to provide the tissue with its unique and complex structure and mechanical properties. Injury or degeneration of cartilage within joints affects millions of people. Healing process is dictated by chondrocytes, which manufacture the ECM. However, the healing response does not lead to the replacement of the appropriate collagen types and proteoglycans. This will result in abnormal tissue functions. Particularly, articular cartilage is difficult to regenerate because of the low mitotic activity of the chondrocytes. This is also due to the avascular nature of the cartilage. Even under ideal conditions, such as when the defect penetrates into subchondral bone, cartilage repair is still difficult because of the slow rate of chondrocytes proliferation compared to scar tissue (Mankin *et al.* 2000a,b).

Currently, loss of articular cartilage associated with osteoarthritis is corrected by total joint replacement, prosthetic joint replacement, abrasion athroplasty, drilling and autologous chondrocytes implantation (ACI). Unfortunately, no treatment has yet resulted in complete satisfactory restoration of the articular cartilage to a normal state. Synthetic material can be easily produced, but they do not have the same natural properties of the original tissue.

This can lead to the long-term immune response problems. ACI though has been proven successful, this method showed various result and some failed to attempt recovery (Knutsen *et al.* 2004; Ashton *et al.* 2003). Tissue engineering of articular cartilage, in which a biocompatible scaffold will be incorporated with chondrocytes to prepare transplantable tissue-engineered human articular cartilage, may provide a more suitable alternative. Pioneered by Vacanti *et al.* in the mid-1980s (Langer & Vacanti 1993), tissue engineering is known as an interdisciplinary field that applies the principles and method of engineering and life sciences toward the development of biological substitutes that restore, maintain or improve tissue function.

Tissue engineering technology in Malaysia started in 1999 in Universiti Kebangsaan Malaysia (UKM). For half a decade, tissue engineering research laboratory which is located at Faculty of Medicine, Hospital Universiti Kebangsaan Malaysia (HUKM) has evolved rapidly in providing valuable beneficial research findings particularly for future clinical application. We are working on human cartilages mainly nasal septum cartilage (Aminuddin *et al.* 2001; Aminuddin *et al.* 2003; Chua *et al.* 2001; Chua *et al.* 2003a,b; Mazlyzam *et al.* 2001a,b; Ruszymah *et al.* 2001a-d), auricular cartilage (Farah Wahida *et al.* 2004; Nur Adelina *et al.* 2004; Saim *et al.* 2004) and articular cartilage.

Recently, our laboratory has successfully cultured human articular chondrocytes (Munirah *et al.* 2003a) and formed tissue-engineered articular cartilage, which is reliable for replacement of damaged tissue. The studies in human articular cartilage engineering showed encouraging results for human articular cartilage development. Previously, we evaluated the quality of tissue-engineered human articular cartilage using macroscopic and microscopic observation as well as gene expression study (Munirah *et al.* 2003b; Munirah *et al.* 2004).

The objective of this study is to evaluate the quality of tissue-engineered human articular cartilage using immunohistochemistry (IHC) technique.

MATERIALS AND METHODS

Cartilage Harvesting, Neo-cartilage Reconstruction and Implantation

This study was approved by the ethical committee of the Faculty of Medicine, UKM. Human articular cartilage was obtained from excessive tissues from consented patients after joint surgery carried out at HUKM. The samples were placed in sterile normal saline and transported to the Tissue Engineering Laboratory, HUKM. All samples were washed with phosphate buffer saline (PBS) (Gibco, Grand Island, NY) containing 100 μg/ml penicillin and 100 μg/ml streptomycin (Gibco, Grand Island, NY), and were minced and digested with 0.6% of collagenase type II (Gibco, Grand Island, NY) in an orbital incubator (Stuart Scientific, Redhill, UK) at 37°C for 6–12 hours for chondrocytes isolation. The isolated cells were centrifuged and the pellet was then washed 3 times with PBS to remove the remaining enzyme. Cell number and viability were determined by using a haemocytometer and trypan blue dye (Gibco, Grand Island, NY) exclusion method.

Chondrocytes were cultured in 9.6 cm^2 of 6 well-plates (Falcon, Franklin Lakes, NJ) for several passages to obtain optimum number of cells. All cultures were maintained in CO_2 incubator with 5% of CO_2 and 95% of humidified atmosphere (Jouan, Duguay Trouin, SH) at 37°C with the media changed twice a week. Chondrocytes were then passaged in 175 cm^2 culture flasks (Falcon, Franklin Lakes, NJ) at a density of 5,000 cells/cm^2 as a large-scale culture expansion to acquire much more cells for three-dimensional neocartilage reconstruction using fibrin.

When the cultures reached confluency, the cells were harvested by trypsinisation using Trypsin-EDTA (Gibco, Grand Island, NY). The cells were washed 3 times with PBS, centrifuged and the resuspended pellet was then transformed into three-dimensional structure using autologous fibrin (Ruszymah 2004). The resulting *in vitro* constructs were then implanted subcutaneously on the dorsal of the athymic nude mice. Specimens were harvested 8 weeks after transplantation. Specimens were then observed macroscopically and processed for IHC evaluation. Normal articular cartilage sections were used as control.

Immunohistochemical (IHC) Analysis

Specimens were fixed in 10% formalin buffer, embedded in paraffin and sectioned using LEICA microtome. The sections were deparaffinised and were then assigned for IHC staining procedures according to DAKO Cytomation Immunohistochemistry staining kit protocol. Tissue sections were pre-treated with proteinase K at 37°C for 60 minutes and washed 3 times with tris buffered saline (TBS) (DAKO Cytomation). All sections were then treated with peroxidase block (DAKO Cytomation) at 37°C for 10 minutes prior to incubation with antibody. All antibodies (Sigma Aldrich) – monoclonal antibody mouse anti-human collagen type II, monoclonal antibody mouse anti-human collagen type I and monoclonal antibody mouse anti-human fibrocytes surface protein – were diluted with antibody DILUENT (DAKO Cytomation) and were applied to the sections for 40 minutes at 37°C. After washing with TBS, the sections were applied with peroxidase labelled polymer conjugated to goat anti-mouse Ig; horseradish peroxidase (HRP) (DAKO Cytomation) for 40 minutes at 37°C. After washing step with TBS, the signal was finally visualised as a brownish precipitate using the freshly prepared peroxidase substrate 3,3'-diaminobenzidine (DAB) (DAKO Cytomation). Sections were then counterstained with haematoxylin and mounted in glycerol gel (DAKO Cytomation).

RESULTS

The native articular cartilage sections that were used as control, stained positive against collagen type II [Fig. 1 (A)] and negative for collagen type I [Fig. 2 (A)] as well as fibrocytes surface protein antigen [Fig. 3 (A)]. As shown in Figure 1B, IHC staining against collagen type II for *in vitro* construct stained positive but with weak expression. *In vivo* construct [Fig. 1 (C)] showed evidence of the synthesis of collagen type II as it is confirmed by the strong immunopositivity of IHC stains consistent with the native tissue [Fig. 1 (A)].

On the contrary, collagen type I which was not present in native cartilage was strongly expressed in the *in vitro* construct [Fig. 2 (B)] and was still detectable in the *in vivo* construct [Fig. 2 (C)]. Fibrocytes surface protein which was also not expressed by native cartilage [Fig. 3 (A)] was stained positive on both *in vitro* construct [Fig. 3 (B)] and *in vivo* construct [Fig. 3 (C)].

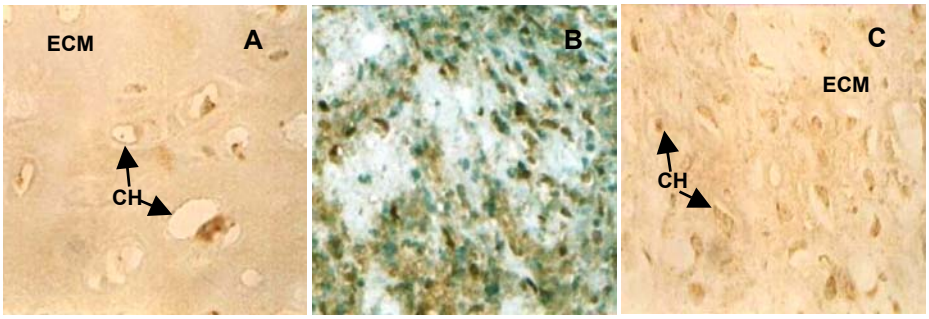

Figure 1: IHC staining against collagen type II (X400). IHC staining on native cartilage (A) exhibits positive expression identify by brown precipitation in cartilage extracellular matrix (ECM), while *in vitro* construct (B) shows typical pericellular expression of collagen type II and noted that *in vivo* construct (C) confirmed the presence or re-expression of collagen type II in consistency to native tissue. "CH" is chondrocytes.

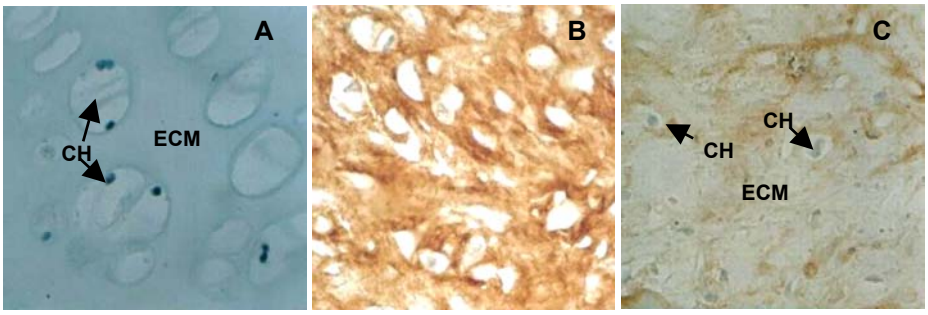

Figure 2: Results of IHC staining against collagen type I (X400) is identified by brown precipitation. Native cartilage (A) illustrates negative expression, whilst *in vitro* construct (B) and *in vivo* construct (C) demonstrate intense immunopositivity of collagen type I. "ECM" is extracellular matrix and "CH" is chondrocytes.

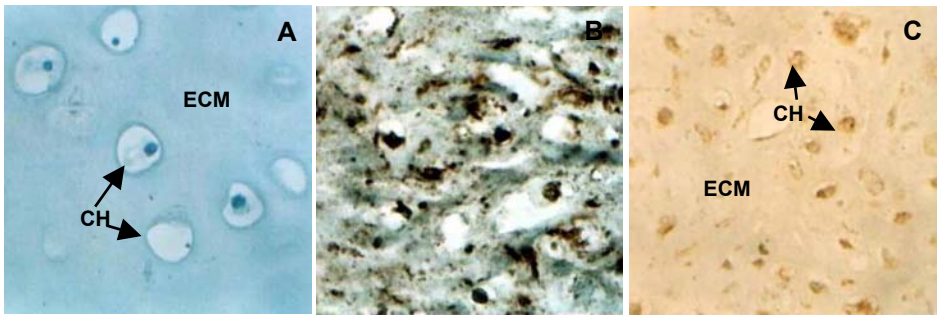

Figure 3: Fibrocytes surface protein (X400) is negatively expressed in native cartilage (A). However its expression is detectable in *in vitro* construct (B) and *in vivo* construct (C) which are identify by brown precipitation. "ECM" are extracellular matrix and "CH" is chondrocytes.

DISCUSSION

Hyaline cartilage, when damaged, has limited ability to repair itself. It has been suggested that this is due to the inability of the chondrocytes to migrate to the damaged area and to regenerate cartilage matrix. It is also believed that poor blood supply may be a contributing factor (Akeson *et al.* 2001). Clinical approaches on treating joint damage include total joint replacement, prosthetic joint replacement, abrasion athroplasty, drilling and a prominent ACI. However, to date there is no treatment outcome results in satisfactory in treating joint damage. ACI is a well-established technique for the treatment of large articular cartilage defects (Peterson *et al.* 2002). Some complications which have been reported with this technique includes periosteal hypertrophy and intra-articular adhesions (Peterson *et al.* 2000).

Scientists and orthopaedic surgeons are now starting to discover an alternative resurfacing technique for cartilage repair. This has aspired tissue engineering to aim at *in vitro* cartilage fabrication with the incorporation of biodegradable scaffold in order to improve the structural and biological properties of the graft, in addition to make it more stable for implantation. We have reconstructed human articular cartilage and proved that the *in vivo* construct expressed collagen type II similar to native hyaline cartilage.

Previously, we have reported the evaluation of the quality of tissue-engineered human articular cartilage by macroscopic observation, standard histological study as well as gene expression analysis using one step Reverse Transcription – Polymerase Chain Reaction technique (Munirah *et al.* 2003b Azmi *et al.* 2004; Badrul *et al.* 2004; Samsudin *et al.* 2004). Macroscopic and microscopic evaluation documented that tissue-engineered human articular cartilage resembled to that of normal hyaline cartilage.

Our three-dimensional *in vitro* construct showed intense immunopositivity of collagen type I and fibrocytes surface protein. Collagen type II is poorly expressed and typically pericellular. Under normal circumstances, both collagen

type I and fibrocytes surface protein are not expressed in native hyaline cartilage. However, in tissue-engineered human articular cartilage instead of the expression of collagen type II, both collagen type I and fibrocytes surface protein are still noticeable on the stained sections.

This analysis showed that chondrocytes are able to express collagen type II gene, a chondrocytes-specific gene after implantation. On the other hand, expression of collagen type I gene in *in vivo* bioengineered cartilage indicated that the engineered tissue is still in the process of development towards mature cartilage (Sasano *et al.* 1996), since 8 weeks *in vivo* incubation is not enough for the construct to fully regain mature cartilage characteristics. However, the results obtained by IHC analysis verified that the chondrocytes in *in vivo* construct or tissue-engineered human articular cartilage has the capability to produce its major component closely resembles to that of native hyaline cartilage.

CONCLUSION

We have successfully engineered human articular cartilage, which resembles the native human articular cartilage; by IHC study. This observation laid emphasis on the rationale of using tissue-engineered human articular cartilage implant for the treatment of articular cartilage defects.

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