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Effectiveness of Natural Membrane as a Scaffold on Chondrocyte Phenotype

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Abstract: Tissue engineering is a new approach to repair cartilage lesions. Autologous Chondrocyte Transplantation (ACT) is one of the tissue engineering methods with some limitations due to expanding of chondrocytes in monolayer culture. Culturing of chondrocytes in scaffold preserves chondrocyte morphology. Extracellular matrix of natural tissues is more applicable to synthetic type of scaffolds in clinic. Therefore, in this study, the amniotic membranes are used as a natural scaffold. In the case of formation cartilage tissue, natural scaffold may be free of problems compared to other synthetic available scaffolds for clinical applications. Samples of human articular cartilage tissue were obtained by surgery. After enzymatic removal, chondrocytes were cultured for proliferation. For tissue formation, chondrocytes were cultured between layers of amniotic membranes for 21 days and examined histologically with H&E, toluidine blue and immunohistochemical staining. The construed tissues in experimental groups were compared with tissues composed of merely chondrocytes and membranes as a control group. Our histologic evaluation of constructs showed that tissue formation has been occurred. Proteoglycan production was observed in cell micromass cultures and cell-scaffold constructs. Immunostaining Method revealed the ECM of cell micromass cultures contains higher collagen type I than higher collagen type II. In cell-scaffold constructs the expression of collagen type I extensively decreased and in this cultures collagen collagen type II expressed more than cell micromass cultures. The current study indicates that application of natural scaffold improves chondrocyte phenotype by maintaining collagen type II expression and inhibiting collagen type I.

Key words: Amniotic membrane, cartilage, chondrocyte, extracellular matrix, scaffold, tissue engineering

INTRODUCTION

Articular cartilage defects are caused by aging, trauma and diseases such as osteoarthritis, posing serious clinical problems. Articular cartilage has limited intrinsic regeneration and self-repair capacity due to its innate avascular nature and low cell to matrix ratio (Diaz-Prado *et al*., 2010; Li *et al*., 2005; Jia *et al*., 2012). Over 151 million people suffer from OA worldwide, representing a huge clinical and socioeconomic burden (Benders *et al*., 2013). Current treatments have been developed to repair articular cartilage defects such as arthroplasty, subchondral drilling, micro-fracture and prosthetic joint replacement. All of the so-called techniques result in fibro-cartilage formation (Kock *et al*., 2012) with fairly acceptable clinical results without functional hyaline cartilage construction (Diaz-Prado *et al*., 2010; Li *et al*., 2012).

In recent years, one of the cartilage tissue engineering methods for treating articular cartilage defects is Autologous Chondrocyte Ttransplantation (ACT) (Chung and Burdick, 2008). However, studies show that this method has some limitations such as dedifferentiation of chondrocyte expansion during monolayer culture. Dedifferentiation is characterized by reduced type II collagen gene expression and proteoglycan synthesis, enhanced type I collagen gene expression and fibrocartilage tissue formation (Chung and Burdick, 2008). According to studies, chondrocyte culture on 3D environment is more efficient than in monolayer culture. In fact, scaffold provides 3D structure for cells like *in vivo* condition and the cells maintain their differentiated phenotype which has a vital role in cartilage tissue engineering (Jia *et al*., 2012). Natural and synthetic scaffolds have been used for cartilage tissue construction *in vitro* (Yang *et al*., 2008). However, so far, none of the scaffolds has been ideal for clinical application. Among scaffolds in tissue engineering, collagen membrane has been proven. Collagen is produced in human tissue, including articular cartilage. Previous studies have demonstrated that scaffolds derived from decellularized ECM and tissues have been successfully used in tissue

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engineering (Yang *et al*., 2008; Chan and Leong, 2008). There is an increasing interest for application of decellularized ECM in tissue engineering because it can provide natural signals for cells. Amniotic membrane has been suggested as a scaffold in fibroblast culture (Yang *et al*., 2009). Fibroblasts adhere to human amniotic membrane and properly proliferate (Kumar *et al*., 2003). Also, human chondrocytes proliferate and reach confluency on the chorionic and epithelial sides of the amniotic membrane (Diaz-Prado *et al*., 2010). Considering amniotic membrane's advantages including its availability, harvesting easily and importantly its biocompatibility, in this study, we hypothesized that amniotic membrane is an appropriate natural scaffold in cartilage tissue engineering.

MATERIALS AND METHODS

Harvesting of articular cartilage: In this experimental study, human articular cartilage is obtained via artroscopy from three knee joints of adult patients with informed consent. Samples were transferred to the cell culture laboratory. After weighing the cartilage tissue under sterile conditions, cartilage tissues were cut into small (1-2 mm) pieces. Chopped cartilage tissues were washed by phosphate buffered saline (PBS, Sigma, USA).

Harvesting and providing of amniotic membrane: Human amniotic membrane was obtained from human placenta by cesarean-sectionwith the mother's informed consent. Under sterile conditions, the amniotic membrane was separated from the placenta chorion. Then, the amnion was washed four times with PBS to eliminate blood and other wastes. Then amniotic membranes were cut into 0.5×0.5 cm size and then they were decellulrized by 0.1% triton X-100 for 30 min.

Isolation and culture of cells: For chondrocyte isolation, the cartilage tissue fragments were digested with PBS and 2.5 % pronase (sigma, USA) for 1 h and then with 0.125% collagenase 2 (Gibco, USA) for 6-7 h in shaking water bath at 37°C. For neutralizing the enzyme, the same volume of medium DMEM (Gibco, USA) containing 10% FBS (sigma, USA) and 1% penicillin/streptomycine (Gibco, USA) were added. The obtained cell suspension was washed by centrifuging in 1600 rpm for 10 min and it was added the medium containing DMEM, 10% FBS, 1% penicillin/streptomycin and ascorbic acid (0.05 mg mL^{-1}). Afterwards, the cell suspension was transferred into flasks and cultured in an incubator at 37°C, 88% humidity and 5% CO_2 for 2-6 weeks. The culture medium was replaced every 3-4 days. After 80% confluency, the cells were passaged.

For tissue formation and accumulation of synthesized extracellular matrix by chondeocytes, the passaged chondrocytes (passage 3-6) were seeded between layers of decellulrized amniotic membrane. Experimental cultures consist of five layers from the decellulrized amniotic membrane (0.5×0.5 cm) and four layers from the suspension of chondrocytes including 10^5 cells. Amniotic membranes (five layers) and 4×10^5 chondrocytes in similar micromass culture were cultured distinctly as control cultures for 3 weeks.

Histological and immunohistochemical staining: After the experimental period, tissue-like cultures were processed histologically for Hematoxylin and Eosin staining (H&E), toluidine blue staining and also Immunohistochemical (IHC) staining. H&E staining: paraffin sections were deparaffinized, rehydrated and stained by Hematoxalin for 15-20 min and Eosin for 10-15 min. Toluidine blue staining: after deparaffinizing and rehydrating, tissue sections (3-5 μm-thick) were stained by hematoxalin for 15-20 min and then by toluidine blue 0.2% for 1-2 min.

Immunohistochemistry staining: after deparaffinizing and rehydrating, tissue sections (3-5 μm thick) were fixed by acetone for 5 min. For blocking endogenous hydrogen peroxidase, the samples were placed in 3% solution of H_2O_2 for 10 min. Proteinase K 10 μL mL^{-1} was used for antigen retrieval for 30 min at 37°C. The samples were then blocked non-specific sites using 10% mouse serum for 30 min at room temperature and then incubated with 1:100 of anti-collagen I antibody (ab6308, USA) and 1:100 of anti-collagen II antibody (ab3092, USA) overnight in a humidified environment at 4°C to detect the existence of collagen types I and II. The secondary antibody was used (ab6563, USA, 1:100) for 1 h in a humidified environment at 37°C. Finally, the sections were developed with DAB; peroxidase was used to define antigen-antibody interaction for 1 h. Slices were counterstained with Hematoxylin for 20 sec and were subsequently dehydrated and soaked in xylene before adding the mounting. Positive and negative controls were included in the immunohistochemistry staining protocol for each antibody. Bone and cartilage tissues are used for collagen type I and primary antibody and for collagen type II, respectively. All samples were observed under an inverted microscope.

RESULTS

In this study, we evaluated effectiveness of natural scaffold on maintaining of chondrocyte molecular phenotype and type of collagen fibers in the matrix. The

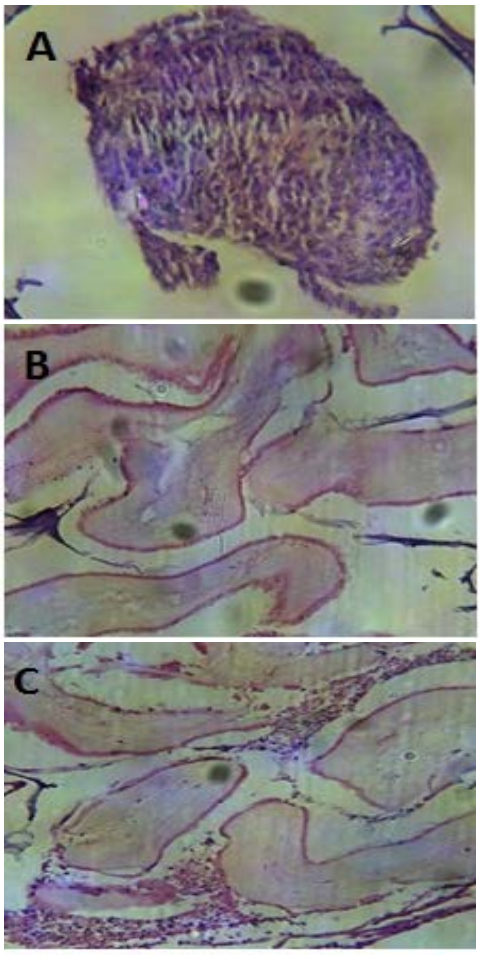

Fig. 1: Micrographs showing constructed tissue by Hematoxylin&Eosin (H&E) staining: A) micromass culture of chondrocytes as control group; ×100; B) amniotic membranes as control group; ×100; C) chondrocyte culture between amniotic membranes as experimental group ×100

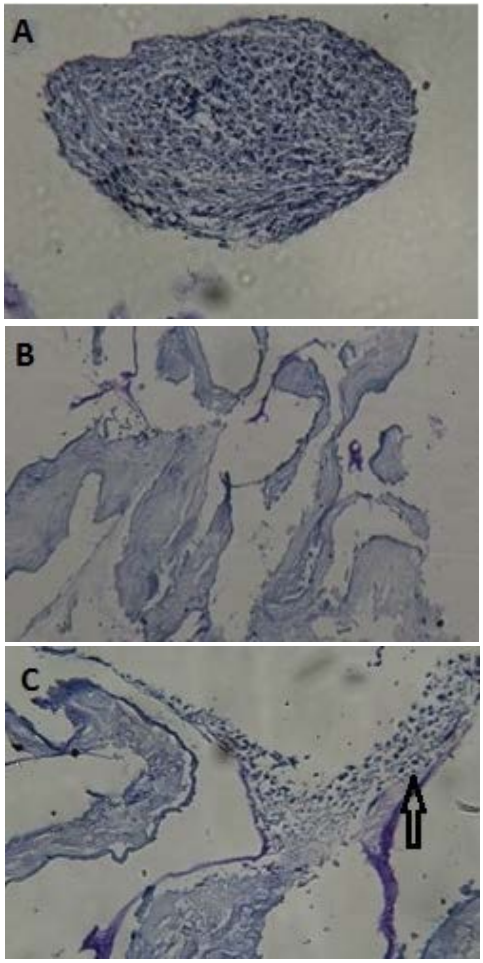

Fig. 2: Micrographs showing constructed tissue with toluidine blue staining: A) micromass culture of chondrocytes as control group; ×100; B) amniotic membranes as control group; ×100; C) chondrocyte culture between amniotic membranes as experimental group, lacunae were indicated by arrows in figure ×100

construed tissues in experimental group were compared with micromass tissues composed of chondrocytes and decellularized amniotic membrane as a control groups. After 21 day, the culture of chondrocytes with or without membrane showed integrated tissue structure. Newly formed tissues were examined histologically by H&E staining for control cultures (Fig. 1A and B) and experimental groups (Fig. 1C). Cultured chondrocytes between layers of amniotic membranes in experimental group produced extracellular matrix that indicates tissue information by H&E staining. Also the constructed tissues were evaluated histologically in order to determine proteoglycans in the extracellular matrix of cartilage (Fig. 2). Experimental group including cultured chondrocytes between of amniotic membranes layers were stained by toluidine blue and we observed proteoglycans formation in the extracellular matrix of cultured chondrocytes (Fig. 2C). Also round morphology of chondrocytes similar to native chondrocytes with lacunae appearance were revealed in newly formed tissue between membranes (Fig. 2C). We observed more collagen type I protein expression in micromass cultures of control group (Fig. 3A) than chondrocyte plus membrane group (Fig. 3C) by immunohistochemistry staining. The ECM of cultured chondrocyte between of amniotic membrane layers in experimental group decreased the expression of

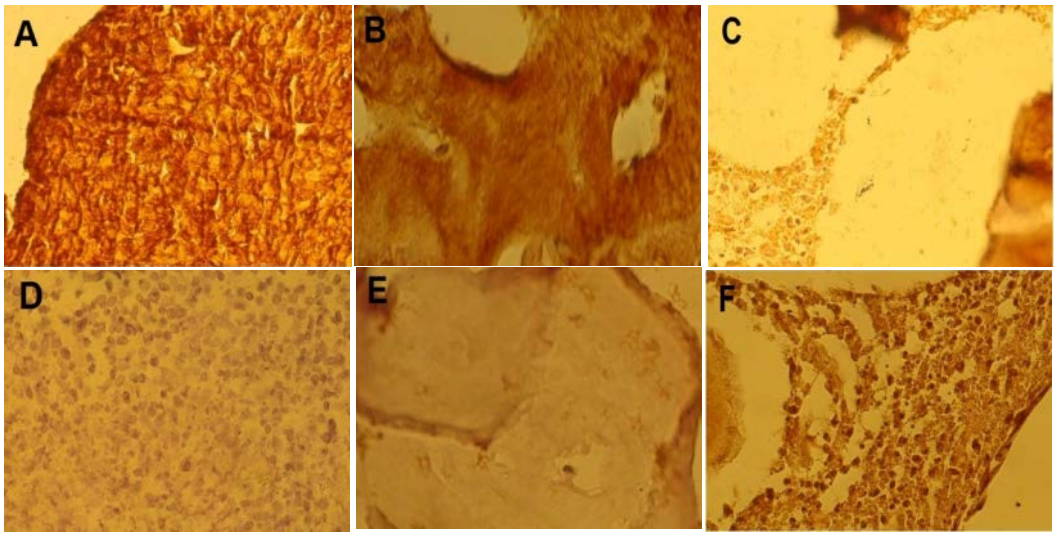

Fig. 3: Collagen type I and collagen type II immunostaining of chondrocyte culture: A) collagen type I staining on control group (micromass culture of chondrocytes): Higher collagen type I was observed in Extracellular Matrix (ECM); ×400; B) collagen type I staining on control group (amniotic membrane): collagen type I was observed; ×400; C) collagen type I staining on experimental group (chondrocyte and amniotic membrane): lesser collagen type I was observed in ECM; ×400; D) collagen type II staining on control group (micromass culture of chondrocytes): lesser collagen type II in ECM; ×400; E) collagen type II staining on control group (amniotic membrane): no collagen type II was observed; ×400; F) in experimental group (chondrocyte and amniotic membrane) strong collagen type II staining was observed in ECM of chondrocytes than micromass cultures ×400

collagen type I extensively (Fig. 3C). Collagen type II protein expressed in cell micromass cultures (Fig. 3D) lower than experimental group (Fig. 3F). Control membranes showed collagen type I staining (Fig. 3B) with any collagen type II protein expression (Fig. 3E).

DISCUSSION

Cartilage defects' restoration is challenging in orthopedic surgery because the mature articular cartilage is avascular and has limited repair ability on injury. Many attempts have been developed to treat such lesions both *in vitro* and *in vivo*. However, each of these methods has some limitations (Hunziker, 1999; Buckwalter, 2002; Detterline *et al*., 2005; Campbell and Pei, 2012; Kleemann *et al*., 2007; Steadman *et al*., 2001). Although, tissue engineering has created new hopes but the efficient production of cartilage tissue and successful treatments are still the main clinical challenges (Keeney *et al*., 2011). The current study set to evaluate the effectiveness of ECM of amniotic membrane as natural scaffold on chondrocyte phenotype. In this regard, biocompatibility of allogenic decalcified bone matrix as a natural scaffold has been shown by histologic methods (Dong *et al*., 2004). Since, the morphology of chondrocytes is spherical *in vivo* but it is flattened *in vitro* to lose their phenotype in monolayer culture due to gene expression changes (Benya and Shaffer, 1982), hence, it is a necessary ideal scaffold to maintain the phenotype of chondrocytes (Temenoff and Mikos, 2000). The scaffold is one of the main elements in tissue engineering. According to studies, natural scaffolds are functionally better than synthetics because they prepare an Extra Cellular Matrix (ECM) and signals that simplify the cell attachment. The extracellular matrix of organs and the decellulared tissues have been used successfully as a scaffold for restoration of various tissues in preclinical and clinical studies. We found that amniotic membrane collagen type I fiber is suitable as scaffold. These scaffolds have excellent biocompatibility. However, *in vivo* studies are necessary to show biocompatibility of amniotic membrane in cartilage tissue. Yang *et al*. (2008) reported that scaffolds from ECM of cartilage may be utilized as biomaterial for cartilage tissue engineering *in vitro* and *in vivo*.

Others used different tissues such as submucosa of the small intestine,decelllarized nerve allografts, heart valves and liver-derived biomatrix as a scaffold for the similar tissue repair. While amniotic membrane has been suggested as a good scaffold in tissue engineering, we applied this membrane for cartilage tissue engineering.

Mligiliche reported that the extracellular matrix of human amnion is an efficient scaffold for peripheral nerve regeneration. Human chondrocytes were proliferated and reached confluency on the chorionic and epithelial sides of the amniotic membrane. Thus, amniotic membrane is low cost, safe and favorable ECM as scaffold for cartilage tissue engineering, hence, the decellularizedamniotic membrane was used as a natural scaffold. In addition as human amniotic membrane cells display some characteristic properties of stem cells, culturing with chondrocytes probably induces these stem cells to chondrogenic cells. However, in this study, we remove amniotic cells of membrane for dispose of immunologic response in heterologous applications for future cartilage tissue engineering. In another study, chondrocytes cultured on amniotic membrane (Diaz-Prado *et al*., 2010) and present study used previously expanded chondrocytes between decellulraized membranes. The result of H&E staining showed that chondrocytes and decellularized amniotic membranes layers formed an integrated tissue and produced extracellular matrix. Also, we showed proteoglycans production in ECM, using toluidine blue staining.

Furthermore, we revealed that the ECM of cell micromass cultures contains higher collagen type I than collagen type II by immunohistochemistry. Also, chondrocytes cultured on decellularized amniotic membranes expressed collagen collagen type II more than cell micromass cultures; however, collagen type I extensively decreased. Accordingly, human chondrocytes seeded on decellularized amniotic membranes could proliferate and maintain their phenotype and could synthesize extracellular matrix of articular cartilage.

CONCLUSION

The current study indicates that the application of natural scaffold improves chondrocyte phenotype by maintaining collagen type II expression and inhibiting collagen type I production. However, further evaluations are required to be done to optimize the usage of natural membranes in cartilage tissue engineering for future clinical applications.

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