

Preparation, Physical and Biological Evaluation of Silk Fibroin/Hyaluronic Acid Scaffold Incorporated with Umbilical Cord Mesenchymal Stem Cell for Cartilage Tissue Engineering

Jirayut Jaipaew

A Thesis Submitted in Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Engineering

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Thesis Title	Preparation, Physical and Biological Evaluation of Silk Fibroin/Hyaluronic Acid Scaffold Incorporated with Umbilical Cord Mesenchymal Stem Cell for Cartilage Tissue Engineering
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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

.....Signature

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Candidate

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Abstract

Silk fibroin (SF) and hyaluronic acid (HA) are both notable biomacromolecules for tissue scaffolds fabrication. SF is biocompatible and can support cell tissue growth. HA can interact with collagen to promote extracellular matrix assembly. Furthermore, HA can interact cell through their receptor such as CD44. The objective of this study was to evaluate the suitability of SF/HA scaffold with stem cells as a performance biomaterial for cartilage tissue engineering. In this study, the chemically cross-linked SF/HA scaffolds with microporous structures were prepared by freeze-drying the aqueous mixtures of SF and HA indifferent ratio (w/w) (*i.e.*, 100:0, 90:10, 80:20 and 70:30). Morphology scaffolds was observed by scanning electron microscope and their mechanical properties including compressive modulus and swelling behavior were analyzed. In this study, human umbilical cord-derived mesenchymal stem cells (HUMSCs) were isolated and their purity and multipotency were then characterized. These stem cells were seeded in SF/HA scaffold under chondrogenic condition and further characterized the chondrogenic differentiation by real-time polymerase chain reaction, histochemical and also immunohistochemical analysis. Cell proliferation and viability of the HUMSCs in SF/HA scaffolds were determined using resazurin reduction test and fluorescein diacetate (FDA) staining. The SF/HA scaffolds showed regular porous structures. Moreover, those scaffolds representing softness and elastic character with the superior in water absorption and swelling. HUMSCs embedded in SF/HA scaffolds in the present of chondrogenic factors can undergo chondrogenesis analyzed by the significantly increased expression of collagen type II, aggrecan and sox9. After 4 weeks of cultivation, the embedded cells continued viable in standard culture medium and in chondrogenic differentiation medium, particularly in SF80 and SF70 also showed the spherical pre-cartilage condensation formation. These results demonstrated that SF/HA scaffolds provided an appropriate environment for the chondrogenic differentiation of HUMSCs. Interestingly, HUMSCs embedded in biocompatible SF/HA scaffolds are hold promise in cartilage tissue engineering application.

ชื่อวิทยานิพนธ์ การเตรียม การประเมิน ลักษณะทางกายภาพ และชีวภาพของโครงร่างไหม/กรดไฮยาลูโรนิกรวมกับเซลล์ต้นกำเนิดจากสายสะดือสำหรับวิศวกรรมเนื้อเยื่อกระดูกอ่อน

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บทคัดย่อ

โปรตีนไฟโบรอินจากรังไหมและกรดไฮยาลูโรนิก เป็นสารชีวโมเลกุลมวลสูงที่ใช้กันอย่างแพร่หลายเพื่อเตรียมเป็นโครงร่างสำหรับงานวิศวกรรมเนื้อเยื่อ โปรตีนไฟโบรอินเป็นโปรตีนที่มีความเข้ากันได้ทางชีวภาพเมื่อใช้เป็นโครงร่างสำหรับการปลูกถ่ายเข้าร่างกายอีกทั้งยังส่งเสริมการเจริญของเซลล์ ในขณะที่กรดไฮยาลูโรนิกส่งเสริมการสร้างโครงร่างค้ำยันนอกเซลล์ด้วยการจับยึดกับคอลลาเจนและสามารถจับกับตัวรับชนิดซีดี 44 ที่อยู่บนผิวเซลล์ได้อย่างจำเพาะ การทดลองนี้มีวัตถุประสงค์เพื่อศึกษาความเป็นไปได้ในการนำสารชีวโมเลกุลทั้ง สองชนิดมาสร้างเป็นโครงร่างรองรับเซลล์ต้นกำเนิดเพื่อใช้ในงานวิศวกรรมเนื้อเยื่อกระดูกอ่อน วัสดุที่มีรูพรุนจากส่วนผสมระหว่างโปรตีนไฟโบรอินและกรดไฮยาลูโรนิกนั้นได้ถูกเตรียมด้วยการระเหิดของน้ำในส่วนผสมที่ผ่านการแช่เยือกแข็งที่ประกอบขึ้นจากส่วนผสมที่สัดส่วนแตกต่างๆกัน โครงสร้างจุลภาคของวัสดุรองรับเซลล์ถูกวิเคราะห์โดยกล้องจุลทรรศน์อิเลคตรอนชนิดส่องกราด ทั้งนี้วัสดุรองรับเซลล์ที่เตรียมได้ยังมีการศึกษาคุณสมบัติเชิงกลได้แก่ความต้านทานของแรงกดและการซึมซาบของเหลว ในงานวิจัยนี้ได้นำเซลล์ต้นกำเนิดชนิดมีเซนไคม์ที่แยกได้จากสายสะดือทารกแรกเกิดมาใช้เพื่อศึกษาคุณสมบัติทางชีวภาพของวัสดุรองรับเซลล์ที่เตรียมได้ โดยทำการเพาะเลี้ยงเซลล์นี้ในวัสดุรองรับเซลล์ที่เตรียมจากโปรตีนไฟโบรอินและกรดไฮยาลูโรนิกภายใต้สภาวะสำหรับการเพาะเลี้ยงเซลล์กระดูกอ่อน การเปลี่ยนแปลงคุณสมบัติของเซลล์ต้นกำเนิดไปเป็นกระดูกอ่อนในโครงร่างรองรับเซลล์ได้ถูกวิเคราะห์โดยเทคนิคทางจุลกายวิภาคของเนื้อเยื่อและการแสดงออกของยีนที่เกี่ยวข้องกับการเจริญของกระดูกอ่อน ทั้งนี้ยังได้มีการศึกษาการเจริญและความมีชีวิตของเซลล์ในวัสดุรองรับด้วย ผลการทดลองแสดงให้เห็นว่าโครงสร้างจุลภาคของวัสดุรองรับเซลล์มีลักษณะเป็นรูพรุนสม่ำเสมออีกทั้งยังมีความอ่อนนุ่มและยืดหยุ่นเมื่ออยู่ในสภาวะอุ้มน้ำ เซลล์ต้นกำเนิดในวัสดุรองรับมีการเปลี่ยนแปลงไปเป็นเซลล์กระดูกอ่อนซึ่งพิสูจน์ทราบได้จากการแสดงออกของยีนที่เกี่ยวข้องรวมถึงลักษณะทางจุลกายวิภาค นอกจากนี้หลังจากการเพาะเลี้ยงเซลล์ไปสี่สัปดาห์ เซลล์ในโครงร่างยังคงมีชีวิตอยู่ ทั้งในนี้ในบางตัวอย่างเซลล์มีการรวมตัวเป็นกลุ่มก้อนซึ่งเป็นคุณลักษณะพิเศษในการที่เซลล์ต้นกำเนิดจะกลายไปเป็นกระดูกอ่อน การทดลองแสดงให้เห็นว่าโครงร่างรองรับเซลล์ที่เตรียมได้นั้นส่งเสริมการเปลี่ยนแปลงของเซลล์ต้นกำเนิดไปเป็นเซลล์กระดูกอ่อน ดังนั้นการใช้โครงร่างรองรับเซลล์จากโปรตีนไฟโบรอินและกรดไฮยาลูโรนิกร่วมกับเซลล์ต้นกำเนิดสามารถที่จะหวังผลดีได้สำหรับงานวิศวกรรมเนื้อเยื่อกระดูกอ่อน

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CONTENTS

Abstract	v
Acknowledgement	vii
Contents	viii
List of tables	ix
List of figures	x
List of abbreviations and symbols	xii
1.Introduction	1
2. Literature review	5
3. Materials and methods	43
4. Results	53
5. Discussion	72
6. Conclusion	76
References	77
Vitae	93

LIST OF TABLES

Table 1. Composition and biophysical properties of silk derived from *Bombyx mori* 35

Table 2. Biomedical Applications of Fibroin-Based Materials 36

Table 3. Biomedical Applications of Hyaluronic Acid 40

Table 4. Primers and their sequences for quantitative real-time PCR analyses 52

Table 5. Mechanical properties of chemical cross-linked SF/HA scaffolds after swelling in PBS 58

Table 6. Swelling ratio and water uptake (%) of SF/HA scaffold in PBS 59

LIST OF FIGURES

Figure 1. The organization of normal articular cartilage. 7

Figure 2. Differences in the physiologic environment and cellular make-up of bone and cartilage have profound effects on the potential to engineer these tissues. 9

Figure 3. Cartilage repair with the microfracture technique 13

Figure 4. Algorithm for treatment of cartilage defects. 14

Figure 5. Schematic diagram showing the different stages involved in the process of autologous chondrocyte implantation 16

Figure 6. MACI uses chondrocytes that have been seeded into a collagen scaffold and cultured for a period of time prior to surgical implantation 20

Figure 7. Tissue engineering techniques for treating cartilage lesions. 24

Figure 8. Neotissue can be formed by combining particulated native cartilage with fibrin glue 29

Figure 9. Scaffoldless tissue engineering. 33

Figure 10. Structure of the repeating disaccharide unit of hyaluronan 39

Figure 11. Regenerated fibroin preparation and fabrication of SF/HA scaffolds. 45

Figure 12. HUMSCs isolation and expansion. 48

Figure 13. Scaffold morphology observed by SEM after treatment with methanol 56

Figure 14. Pore sizes of the SF scaffolds 57

Figure 15. The multipotency of HUMSCs in this study 60

Figure 16. HUMSCS proliferation in the different types of silk/HA scaffolds 63

Figure 17**.** Fluorescence micrographs of HUMSCs cultured on silk/HA scaffolds 64

Figure 18. Cell viability during chondrogenesis 65

LIST OF FIGURES (CONT.)

Figure 19. H&E and alcian blue staining. Chondrogenic differentiation of pellet cultured of HUMSCs 67

Figure 20. Immunohistological staining for the expression of collagen type II of induced HUMSCs 68

Figure 21. Immunohistological staining for the expression of aggrecan of induced HUMSCs 69

Figure 22. Relative normalized expression of chondrogenic-specific gene 70

LIST OF ABBREVIATIONS

Aggrecan-degrading enzyme disintegrin, metalloproteinase with thrombospondin motifs 5 (ADAMTS-5)

Autologous chondrocyte implantation (ACI)

Autologous matrix

Chemokines (CCL)

Cluster of differentiation (CD)

Extracellular matrix (ECM)

Fibroblast growth factors (fgfs

Fibroblast growth factors receptor (FGFR)

Glyceraldehydes-3-phosphate dehydrogenase (GAPDH)

Human umbilical cord-derived mscs (humscs)

Hyaluronic acid (HA)

Interleukin (IL)

International Knee Documentation Committee (IKDC)

Matrix metalloproteinase 13 (MMP-13)

Matrix-induced autologous chondrocyte implantation (MACI)

Mesenchymal stem cells (mscs)

N-terminal propeptide (PIIBNP)

Osteoarthritis (OA)

Peripheral blood stem cells (pbscs)

Phosphatidylinositol 3-kinase (PI3K)

Polymerase chain reaction (PCR)

Secreted protein acidic and rich in cysteine (SPARC)

Silk fibroin (SF)

Sulfated glycosaminoglycan (gags)

Three dimensions scaffolds (3Ds)

Tissue inhibitor of metalloproteinases-1 (TIMP-1)

Transforming growth factor beta (TGF

Tumor necrosis factor-inducible gene 6 protein (TSG-6)

CHAPTER 1

INTRODUCTION

The incidence osteoarthritis (OA) associated in aged population or sport-related joint injuries in young population were increase in the past few years. The strategy for the reconstruction of defects or injuries in articular cartilage was greater interested. There are numerous present therapeutic approaches have been developed for regeneration of such injuries, including microfracturing (1, 2) , autologous chondrocyte implantation(3, 4), matrix-induce autologous chondrocyte transplantation(5, 6), and also hyaluronic based scaffolds implantation (7). However, these methods represent the deleterious effect (8) including insufficiency of harvested healthy cartilage, isolation difficulty, limitation of chondrocyte expansion and also chondrogenic dedifferentiation *in vitro* which not provided the optimistic treatment of articular cartilage lesions.

Cells, signals and scaffolds are the major elements of tissue engineering approaches to cartilage repair. For this purpose, many different cell sources (autologous cells, allogeneic cells or stem cells) have been investigated *in vitro* and *in vivo*. The appropriable of scaffolds are also interested for treatment of cartilage injury. Combinations of cells, scaffolds and signal drive the cartilage engineering strategies as scaffold-based implants that promote cell recruitment with chemoattractants; cell-seeded scaffolds that mimic the structure of native tissues.

Previously, fabricated silk fibroin (SF) scaffolds with or without modifications were implicated as biomaterial for cartilage tissue engineering application (9, 10) due to their superior mechanical property when compare to other natural protein biomaterials in the point of structural maintenance during tissue regeneration (11). Although, SF representing most favorable properties for tissue regeneration including chemical, mechanical and also

biological but for the cartilage regeneration it still need to combine with another materials for the improvement of their biological function. Interestingly, hyaluronic acid (HA) is also one most biomaterial which has the extensive study for cartilage tissue regeneration (12). HA can interact with collagen to promote extracellular matrix assembly and also interact with cells through their receptor such as CD44 on plasma membrane (13). However, HA and HA based scaffolds present some limitations including, inadequate mechanical properties and biodegradation (14). Thus, this study was interested to combine those properties of such two biological macrobiomolecules to design the biomimetic chondrogenic support biomaterial. Moreover, this study also interest to use the alternative source of mesenchymal stem cells (MSCs), human umbilical cord-derived MSCs (HUMSCs) to study the *in vitro* function of the fabricated scaffolds

Hypothesis

The hypothesis of this research was based on;

1. SF acts as the main structure and tissue conductive component of the scaffolds, providing mechanically stable structures, while HA creates the elasticity to blend scaffolds and also provide biomimetic surface for mesenchymal stem cell culture and ingrowth.

2. SF/HA scaffolds represents the chondrogenic support for the differentiation of HUMSCs the feasibility for using the scaffolds for cartilage tissue engineering application.

3. Human umbilical cord derived MSCs embedded in SF/HA scaffold has potential to use in cartilage defect especially for osteoarthritis.

Objective

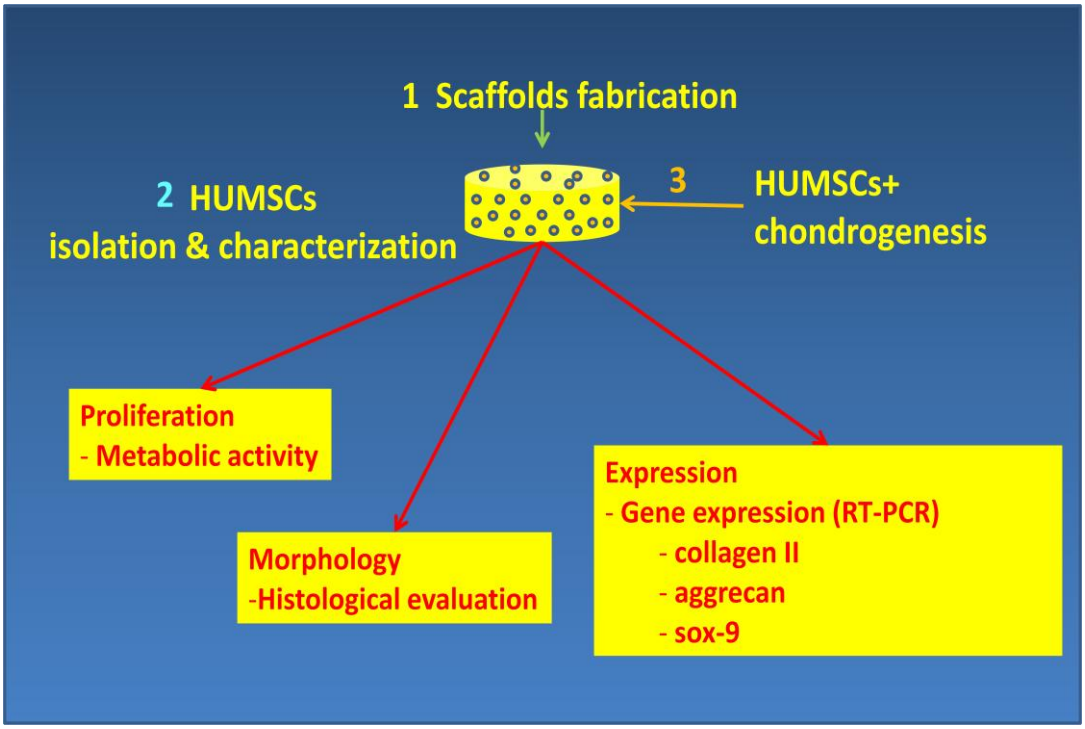
The specific objectives of this study are to:

1. To produce three dimensions scaffolds (3Ds) from silk fibroin (SF) with hyaluronic acid (HA)

2. To investigate the physicochemical, mechanical, and cell supporting properties of 3Ds SF/HA scaffold to determine the suitability of 3Ds SF/HA scaffold as a potential biomaterial for cartilage tissue engineering

3. To create the engineered material SF/HA scaffolds incorporation with HUMSCs for cartilage regeneration.

Experimental outline



CHAPTER 2

LITERATURE REVIEW

2.1. Cartilage and osteoarthritis (OA)

2.1.1 Cartilage structure

Articular cartilage is a connective tissue located at the edges of bones joints and its major function is joint lubrication and protection from the mechanical loading. These tissues consist of chondrocytes scatter in the rich well of extracellular matrix (15, 16). The major extracellur matrix of articular cartilage is including collagen, aggrecan and other proteoglycan. In adult, the architecture of articular cartilage (figure 1) is separated into three zones including, superficial, middle, and deep zones (17). There are varying in extracellur matrix, cells distribution, cell morphology and cell metabolism (16). The superficial zone of the articular cartilage is approximately 10–20% of the full depth. Chondrocytes in this area show flatted, with the elongated morphology and the orientation of cells are parallel to chondral surface which surrounding by the packing of condensed collagen fibers, concentrated with decorin and biglycan but low levels of aggrecan. The superficial zone also has proteoglycan 4, the lubricant and load-bearing protein for chondral area (18). The 40–60% of the cartilage width is transitional or middle zone, approximately, represent the cluster of chondrocytes surrounding with randomly organized of collagen type II fibrils with high concentrations of aggrecan, hyaluronan and dermatan sulfates (15). In the beneath zone, chondrocytes are ellipsoidal, and columns stacking with the specialized pericellular matrix and dispersed by a radial orientation of collagen matrix (16).

2.1.2 Cartilage homeostasis

Articular cartilage homeostasis is gathered by the balancing of aggrecan and collagen which provide as a loader of tensile strength and compressive and joint mobility (15). Aggrecan moieties are non-covalently associated with collagen fibers and inconsuderable conditions such as causing the loss of the cartilage immobility. However, aggrecan were hastily replenished by the residency chondrocytes. On the distinction, human collagen type II in cartilage ECM is a long-lasting protein (19) and their breaking-down, synthesis and maturation processes are complicated and prolonged, and might be key limitation factors for cartilage regeneration. The superficial chondrocytes disintegration by the mechanical joint forces is probably replaced through the activity of chondrogenic progenitor with a homeostatic process. However, the cartilages at transitional and deep zones grant the slow turnover of matrix and cells.

2.1.3 Cartilage injury

The self remodeling of cartilage is relatively scarce as resulting of the deficiency of blood, nerve progenitor cells and sparsely of embedded chondrocytes. For the osteochondral defect stem cells from bone marrow migrate to the injury site and promote the neocartilage formation however the new tissue is fibrocartilage (figure 2) which cannot restored their normal function especially the mechanical integrity. The joint tissues scar would intervene with the surface smoothened and would have destructive mechanical properties. In the bovine and human *in vitro* cartilaginous wound model, it was demonstrated that chondrocytes in superficial zone fastly proliferated, migrated and spread throughout damaged cartilage in a manner resembling reepithelialization of injures (20). The formation of fibrin clot does not involve in cartilage injury, inflammatory cells infiltration and angiogenesis. As noted, the production of anti-angiogenic constituent in cartilage tissue such as thrombospondin-1, chondromodulin-1, SPARC (secreted protein acidic and rich in cysteine), collagen type II, derived *N*-terminal propeptide (PIIBNP), and the type XVIII derived endostatin (21) which

actively resists the neo-vascularization . Furthermore, calcified cartilage and tidemarks at osteochodral area both are two anatomical vascularization blockade. In OA, there are multiple degradation of tidemarks, as if to beat off the subchondral bone vascularization. In the supplementary of this noted, vascular growth factors injection in the joint pocket induces OA professing its adverse event in the joint (23)

Genes expression analysis by microarray in whole tissues dissected or microdissected of joints of OA models from mouse and other animal, including articular cartilage, meniscus and epiphysis, , representing the advanced in molecular understanding the repairing of cartilage wound. The injury cartilage decides in proposition a studies of molecular events, parallels to the other wounds, such as the proteases expression and the growth factors releasing (24). In response to abrasion, the inflammatory response genes in the damaged cartilage are a specifically activated. The inflammatory gene expression followed through the damaged cartilage are not totally resemble to those effected by cytokines such as interleukin 1α (IL-1α), and they are also liberated of the results of either gene activation or products from joints non-cartilaginous tissues. In the damaged cartilage, three MAP kinases JNK, p38 and extracellular-signal-regulated kinase (ERK) were activated, as well as NF-kB phosphatidylinositol 3-kinase (PI3K) and also Src kinases, (25, 26). Additionally, there are other genes activation in damaged cartilage, including inflammatory cytokine genes and also chemokines such as IL-1α, IL-6 and CCL2 (27). The responses of cartilage damage can be devided into catabolic and anabolic activity. Catabolic activition is the most applicable for cartilage in the releasing of the disintegrin (aggrecan-degrading enzyme), matrix-metalloproteinases, thrombospondin motifs 5 (ADAMTS-5) and the collagenase (matrix metalloproteinase 13, MMP-13), which specifically degrade to type II collagen. ADAMTS-5 is released during early phase of damage and the expression of MMP-13 shows a responsibly delayed (27). In contrary, the anabolic activity including the induction of genes

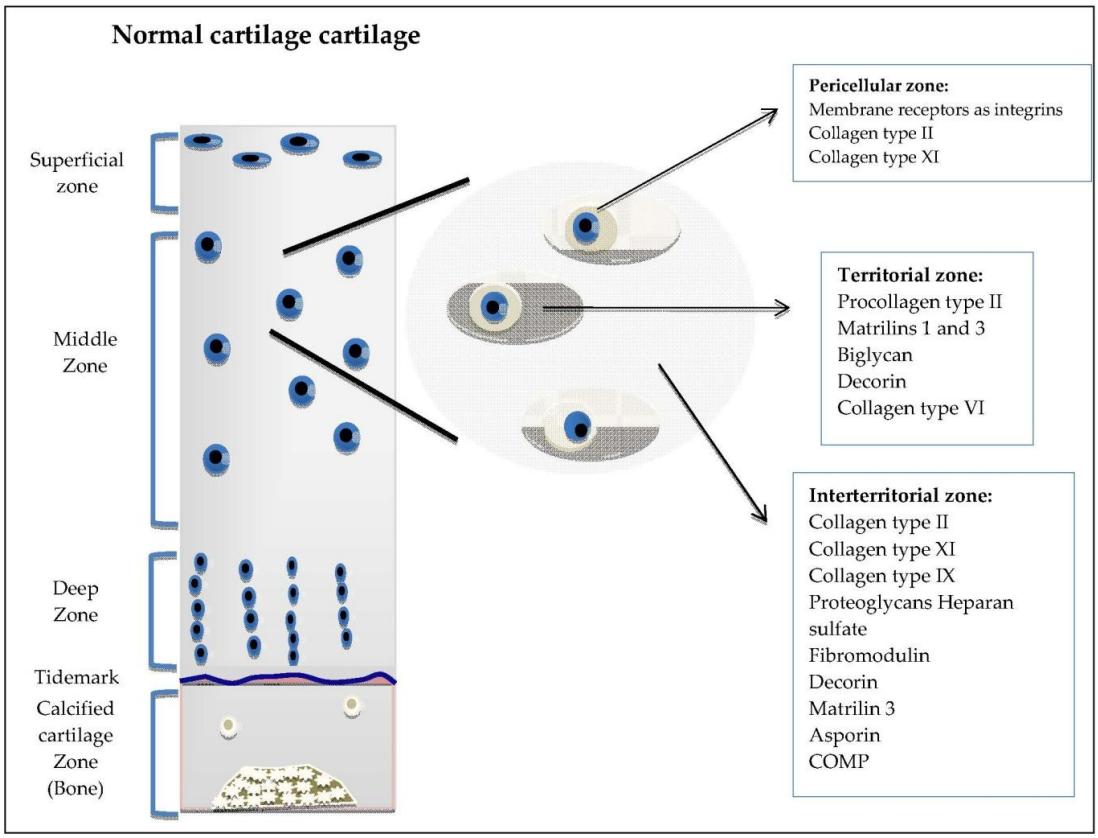


Figure 1. The organization of normal articular cartilage. The organization of chondrocytes is divided in superficial, middle or transitional, deep or radial and calcified cartilage zones with a boundary or tidemark between the first three zones and the calcified zone. The extracellular matrix is divided depending the distance from the chondrocytes. The pericellular zone is the matrix surrounding immediately the chondrocytes. The territorial zone is the next to pericellular zone and the interterritorial zone is the most distant. Every zone has specific characteristics related with the shape of the chondrocyte as well the activity and the expression of different molecules by the cell (22). for chondro-protection such as tumor necrosis factor-inducible gene 6 protein (TSG-6), hyaluronic acid-binding anti-inflammatory moiety, tissue inhibitor of metalloproteinases-1 (TIMP-1) and a transforming growth factor beta (TGF-β) family member (activin A) (27).

2.1.4 Cartilage repair mechanism

The expression of inflammatory responsegenes in cartilage damage were controlled by several mechanisms. Cartilage is a mechanosensible connective tissue and disorientation of ECM could sense in the activation of chondrogenic surface receptors, along with, integrins, calcium ion channels, primary cilium and discoidin receptors domain (27, 29). When the stability of the pericellular ECM is contravened and subjected to compression cycles, resulting in the release of reserved growth factors (29). The pericellular matrix is a specialized ECM that immediately circumscribe chondrocytes and also enriches with nonfibrillar type VI collagen, proteoglycan , perlecan and the heparan sulfate, within pericellular matrix are quarantined by various factors such as fibroblast growth factors (FGFs significantly FGF-2 and FGF-18), growth factor for connective tissue, insulin-like growth factor (IGF) and activin A, bone morhogenetic protein (BMP) and their inhibitors. FGF-2 activated the interaction by FGF receptor (FGFR)-1, promotes degradation of cartilage and FGFR-3 engagement to actuate joint protection (30). In mouse models, FGF-2 interrupts cartilage degeneration. FGF-2 gene knockout mice accelerated the development of OA spontaneously and followed by joint instability(25). In the human cartilage, arrangement of FGF-2/FGFR-1 plays as an competitor by the enhancement in the induction of ADAMTS-5 and MMP-13(30). However, engagement of FGF-18/FGFR-3 is an anabolic results in human articular chondrocytes, reflection by chondrocyte viability increment and therefore the formation cell-associated ECM (30). Additionally, other factors to the TGF proteins family and growth factors, such as Wnt signalling molecules are also delivered followed by cartilage damage (31). The organogenesis of normal cartilage and therefore the development of OA cartilagewould be affected by all of these growth factors. All together, articular cells and the ECM associate in cartilage damage. Firstly, chondrocyte is activated and migrates from their locations in the matrix for the initiating of repairing and regeneration process. The releasing of ADAMTS-5 and MMP-13 is mainly achieved. Reactive oxygen species produced by

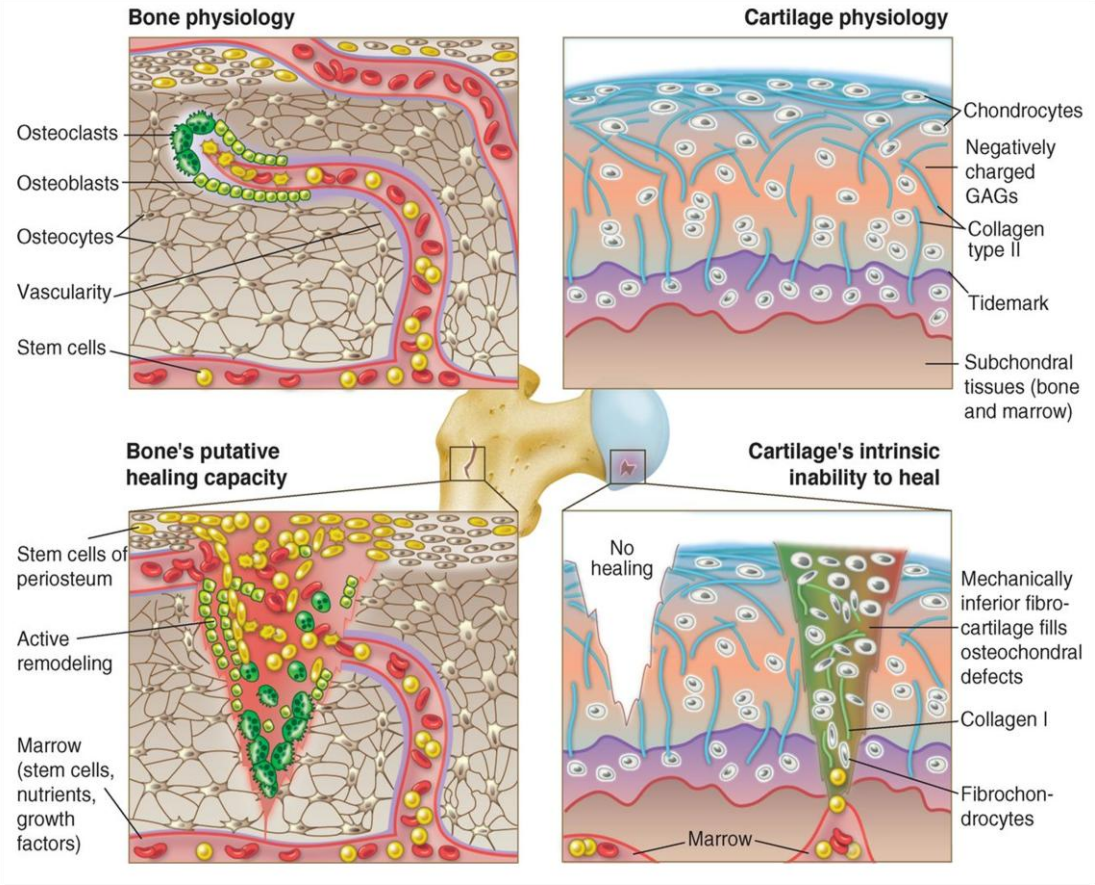


Figure 2. Differences in the physiologic environment and cellular make-up of bone and cartilage have profound effects on the potential to engineer these tissues. Through the presence of stem cells in marrow and in the periosteum, and access to abundant nutrients via vasculature, bone possesses inherent regenerative capability that can be harnessed in regenerative therapies. Cartilage's hypocellularity and lack of nutrient supply, coupled with the inability of bone marrow MSCs or resident chondroprogenitor cells to generate hyaline ECM, result in a tissue unable to mount a functional healing response. Thus, in contrast to bone's ability to heal, cartilage needs more robust exogenous approaches to achieve satisfactory regeneration (28).

activated chondrocytes may also expedite the matrix breakdown(15). When the degradation of damaged tissue occur, progenitor chondrocytes migrate to the damaged site and start the regenerative cascade (15). At superficial zone, the cartilage repairing is rapidly accomplished by chondrocytes in superficial zone. Despite, the disintegration and regeneration of the transition zone to deeper zone of cartilage could be a diminish process. Currently, in general, the regeneration of cartilage is almost offensive in vertebrate. However, it has been examined in a mouse articular cartilage regeneration models and resulted that some mouse strains are robust heritable healings while other strains are non-heritable (32, 33).

Cartilage regeneration appearing in the transition and deep zones with has proven by several experimental studies. For instance, several OA studies have shown that the initiations of matrix synthesis increment and cell growth were taken in both zones. Chondrosphere are assembled in the transition and deep areas of cartilage (34). However, in despite of these correctable processes, in general, there is not materialized in OA for cartilage regrowth . The rationales of cartilage regrowth failure are elusive. The additional evidences were accommodated from cartilage engineering studies; for example, the laterally integrate of neo-cartilage to neighboring cartilage is barely noted (35). Also, healing in OA cartilage of vertical fissures has been fail, indicating of lateral integration failure of OA cartilage tissue disease.

2.2. Recent technique for treatment of osteoarthritis

Microfracture was submitted to the clinic in the late 1980s and early 1990s thereafter other bone marrow stimulating procedures were used in which puncture through area of subchondral bone. This procedure can augmentate MSCs migration from bone marrow to the defect site of a cartilage (Figure 3); nonetheless, microfracture often leads into the formation of fibrocartilage which their biochemical and biomechanical were totally different from hyaline articular cartilage. Several case reports study has shown that, the repaired tissue is

susceptible to forces of mechanical joint without the constitution of mechanical property by hyaline cartilage and deteriorates within 18–24 months typically after surgery. Such particular degradation occurs after large defects treatment or the patellofemoral joint location. Additionally, intra-laceration osteophytes developed in 20–50% of patient attributing to the subchondral bone penetration (36). The alteration of arthritic bone biomechanics could cause a trice-to-septuplicate elaboration of failure of autologous chondrocyte implantation (ACI) when operated after microfracture performed. Specific indications for microfracture technique performing in cartilage lesions are depend on the defect size, deepness and defect location in the joint, likewise the age of patient and BMI (figure 4). Regarding to FDA regulation and orthopedist still acknowledged the gold standard by microfracture for cartilage restoration, eventual parallel studies reveal that microfracture could lag the temporary degeneration of articular cartilage only; surgical failure can be counted disregarding to lesion volume. A detailed of indications confession, clinical application, s of the standard microfracture procedure rehabilitation and outcome has been reviewed elsewhere (37). Despite, there are some important of microfracture understanding , in brief this procedure has become a pervasive approach for regenerated surgery of defected cartilage, the weak points of that have eventually prompted the procedures development of which goal to complete articular cartilage regeneration. The contradictory results of microfracture spurred ACI development. The ACI was perform collected of a full consistence cartilage from a low-weight-bearing area of knee joint by punching biopsy at first-time arthroscopy for the providing of a chondrogenic cell number around 12–48 million cells acquiescence which is then expanded *in vitro*. The pre-cultured chondrocytes are seeded into cartilage lesion after the debridement and bound by a periosteal graft during a second operation (Figure 5). This technique has two major advantages: the using an autologous cells can bypass potentiality immunological complications or infection by viruses from allogenic chondrocyte or foreign biomaterials transplantation; the small biopsy for the chondrocyte harvesting can minimize complications of donor which is opposite to autologous osteochondral cells implantation. Effective of

functional and clinical benefits of ACI have been confirmed by clinical trials. The more than 10years follow-up of long-term case series have revealed that ACI is a resultant and lasting for treatment of large (>4 cm^2) lesions of knee cartilage (40). The similar results have been demonstrated by other clinical trials (41, 42) which giving prominence to the beneficial of the combination a curative osteotomy and ACI (43). Either ACI is better to other approved protocol in prospective randomized clinical controlled trials, after all is still disputable. A trail comparing ACI with mosaicplasty of osteochondral auto-transplantation demonstrated that ACI have better clinical outcomes more than 10 years postoperatively (44); other trials for the treatment of cartilage defects >3 cm^2 in size showed that ACI has better results in comparison with microfracture (45).However, the investigation of ACI and microfracture for smaller lesions representing the similarly evident of clinical and radio graphically outcomes (46), although ACI has been affiliated to the improvement of structural repair. In additional large-degree prospective randomized trials with a long-period follow up are essential for the clinical criteria identification and appraisal , advantage and disadvantage of ACI could accommodate better clinical results over microfracture which was less invasive and expensive procedure. Alluringly, the evaluation of ACI function should be augmented by neotissue characterization with exceptional image analysis or arthroscopy operations. There are three major complications by ACI: two operations require; a long time recovery (6–12 months) for ensuring neotissue formation and maturation and the baseline improvement of clinical scores; and ACI is complex and also a multistage procedure. The hypertrophy of periosteal membrane for cartilage defect sealing of implanted cells thereafter ACI was the most frequently reported adverse event (48). Therefore, alternative procedures by using artificial matrices such as porcine membranes including the combinations of collagen types I and III or hyaluronan matrices. Despite, these biomaterials increase the likeliness of an immunological response to the foreign scaffold, and their usage is recently acknowledged off-description in USA.

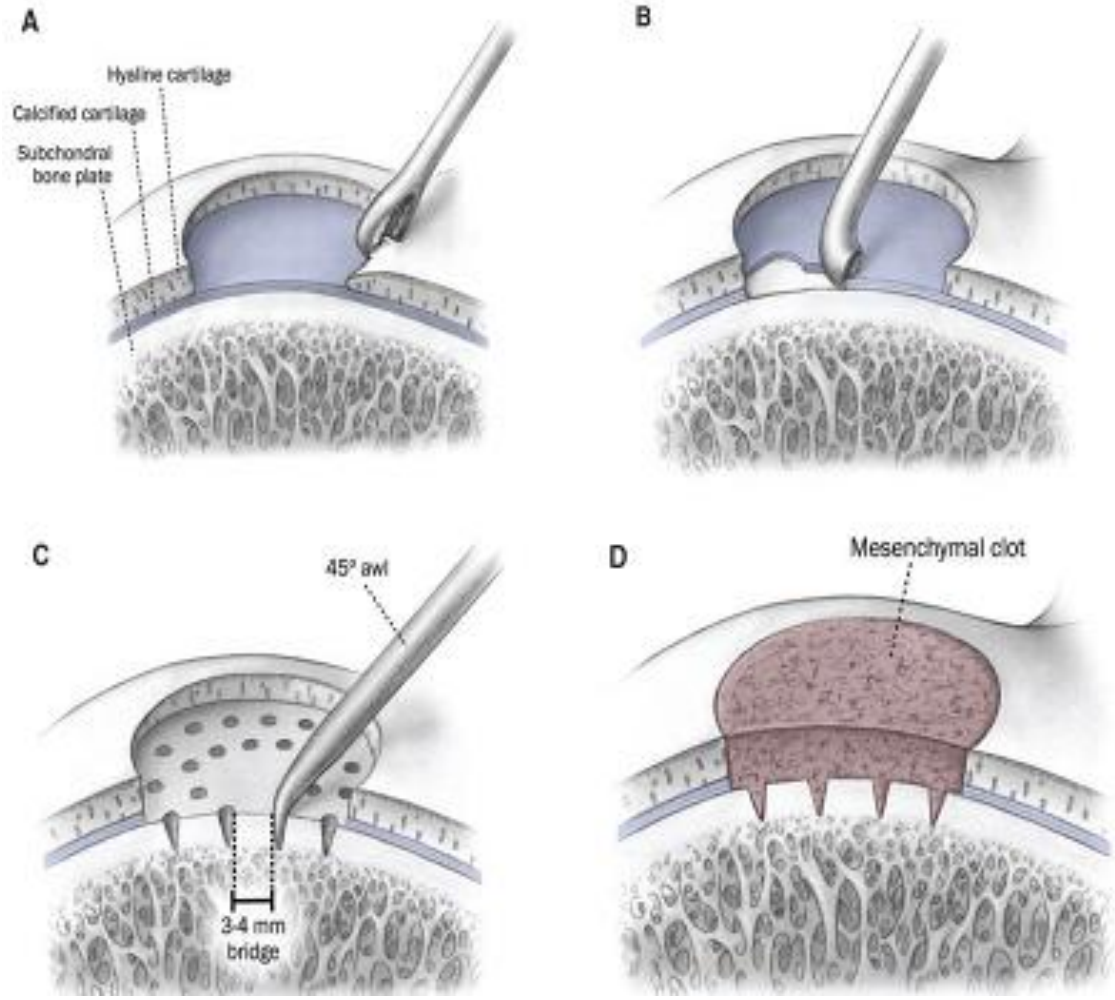


Figure 3. Microfracture technique for cartilage repair involves several systematic steps, including cartilage margin debridement (A), carefully removal layer of the calcified cartilage (B), and microfracture penetrations through the cartilage defect (C), resulting the complete defect fill by a well-imbed mesenchymal clot (D) (38)

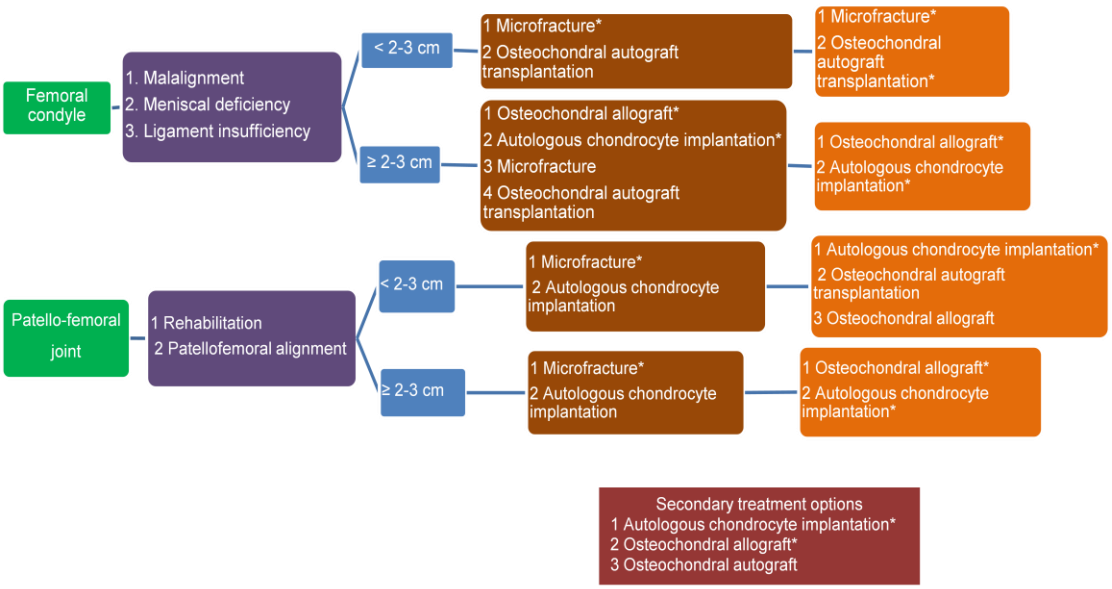


Figure 4. Cartilage defects treatment algorithm. *Recommended treatment options (39).

Elementary reports have also demonstrated the dedifferentiation of autologous chondrocytes ‘’ into fibrochondrocytes in culture (49). However, other work shows theredifferentiation and expression of chondrogenic markers after presence reestablished toward an *in vitro* system by 3D culture (50). To assimilate clinical results of ACI, the generation of a therapeutic algorithm for the direct improvement of a successful outcome have been established by the identification of patient-specific and cartilage defect-specific factors (figure 4). Nonetheless, the cost-effectiveness of ACI is further needed to be investigated by large-scale cohort studies. All over, ACI has demonstrated profitable treatment for cartilage lesions; however more investigation demands to be accomplished for the biomechanical stabilized scaffolds development, to accomplish better assimilation with the host tissue and therefore hastily maturation of the neotissue .

The lack of an ancillary intrinsic guidance of matrix synthesis by scaffolds and organizing could, in part, interest for the consequence inconstancy beyond populations of patient treated with autologous chondrocyte implantation procedure. *Ex vivo* trials demonstrated that the successfully in the regeneration of cartilage is controlled by the chondrocyte differentiation rate and the proliferation capability of MSCs within a 3D scaffolds for tissue engineering, a structure that carry the cell and therefor maintenance and support the critical characteristics of cartilage (51). Therefore a secondary production of ACI has attracted to develop scaffold-based procedure to delivery chondrocytes to lesion site of cartilage. The major advantages of scaffold-based approaches over to scaffold-free techniques: including, the cartilage defect was increased control to better fill; less donor area complications; lesser challenging extent in technically procedure; and increased graft stability due to shorter postoperative recovery time. In addition, as the result of the chondrocytes was cultured into 3D microenvironment, cells dedifferentiation are less decumbent and higher consequently production of hyaline-like cartilage (52). Additionally, chondrocytes culture before transplantation might aid to control the quality of this repairing technique.

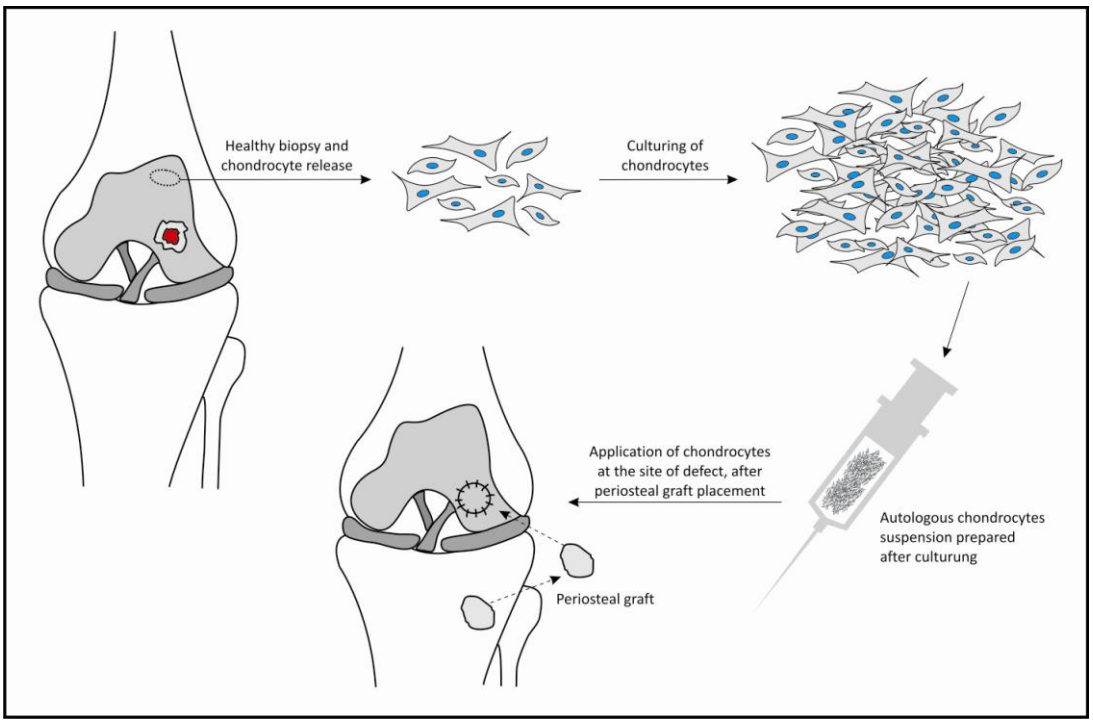


Figure 5. Schematic diagram showing the different stages involved in the process of autologous chondrocyte implantation (47).

There is currently most common clinical use technique for cartilage repair called matrix-induced autologous chondrocyte implantation (MACI) is the scaffold-seeded with cells. Coinciding to ACI, two surgical procedures were required in MACI. The first operation collects autological chondrocytes from low load baring cartilage tissue of patients. Once isolated, chondrocytes was *in vitro* explicated and then cultured for 3 days onto the absorbable membrane of porcine-derived combination of type I and III collagen before implant. These specifically engineered collagens can help chondrogenic penetration on inside and less abrasion on outer part. The chondrocyte-seeded scaffold was implanted by a mini-arthrotomy exposion and defect debridment, after that the scaffold is then fixed by using fibrin glue, the chondrocyte-laden side was facing to subchondral bone and the low-abration part was facing the synovial cavity (Figure 6).

Even though case studies of MACI have had assuring histological and clinical outcomess (5) the ascendancy of MACI beyond current procedures leftovers arguable. MACI was found equivalent clinical, arthroscopic and histological outcomes when compare to ACI by the assessment from one prospective randomized clinical trials (53) however, the hypertrophy rate of the transplant had a lower in MACI-treated group. Another clinical trial reported that undifferences of Tegner activity scores, International Knee Documentation Committee (IKDC) scores, later submission of MACI or ACI by 2 years; despite, Lysholm and Guillquist functionality of knee scores represented superior capacity of ACI procedure over MACI (53). A discrete research demonstrated results of MACI to be greater than microfracture as 2 years cases with large (>4 cm^2) lesions later on surgery (54). All over, these investigationss demonstrated after ≥2 years of MACI have similar outcomes in a follow-up study of either ACI or microfracture. Even though MACI is an adorable technique as indication of safety, reproducible, simplify of surgery and invasion reduction and intra-operative time, the assessment of the procedure assurances the another study. Comprehensive prospective randomized controlled trials with long-term follow-up are further crucial for the

identification of clinical benchmark, advantages and disadvantages for the optimization of technical achievement of MACI.

Hyaluronan-based matrix is intended to use for promotion and maintainance of the chondrogenic phenotype and the synthesis of type II collagen througout *in vitro* culture, resemble to thereafter implant (55). Despite, *ex vivo* investigatios revealed that the rate of degradation of hyaluronan-based scaffolds can be tailor-made equivalent to the synthesis rate of extracellular matrix (ECM) by the chondrocyte to support the combination within the lesion tisssue (55), however, this result has not been accepted for long-term clinical investigations.

Autologous chondrocytes treatment the cartilage defects by hyaluronic-acid-based scaffold delivery are hold capability. As case reports, the hyaline-like tissue were observed after about 1 year implantation (56) and functional improvement and quality of life on the basis health-related results have been published after implantation for 3 years, related to the basis. A prospective cohort study for the evaluation of sport-related patients treated with either microfracture or hyaluronan-based MACI, established comparable outcomes in 2 years postoperatively(56). However, the hyaluronan-based MACI revealed the better IKDC scores than the group treated with microfracture which were discovered at 7.5 years after surgery. Interestingly, athletes receiving hyaluronan-based MACI required 12.5 months for recovery prior turning back to play sport, considering athletes recieved microfracture requisited for 8 months (56). Thus, despite hyaluronan-based MACI eventually have supeior clinical results than microfracture, but request long-term rehab. In a small cohort investigation, there were not statistically significant dissimilarity in the operative results or the tissue histology and immunohistochemical constitution of hyaluronan-based and fibrin-based matrixs at 1 year after operation to implant the scaffolds (58). Despite ACI with scaffold for cartilage repair is accepted in EU, but it currently awaiting for approval by US-FDA, however, completely-

accomplished correlative investigation with long-term follow-up are desired to validate the conceivable therapy and economic account.

2.3. Tissue engineering approaches for treatment of osteoarthritis

The advantages of MACI procedure in clinical trials have demonstrated the benificial functionality were similar to stimulation by mechanical clue was introduced to *in vitro* cell-seeded scaffolds, in mode of either dynamic compression (59) or hydrostatic pressure (60) for the improvement of matrix growth and the function of neotissues. Growth factors that help chondrocytes for characteristic maintenance and capability for ECM production were identified. The transforming growth factor β (TGF-β) family are the crucial growth factors that widely used in *ex vivo* studies of cartilage tissue engineering and regeneration , espacially TGF-β1 and TGF-β3 (61). TGF-β stimulation can help in the maintaining of the native chondrocyte phenotype which have confirmed for *in vitro* tissue engineering studies and therefore, biochemical enhancement on the functional and proportion proprietary of the neo-cartilage, and advocates the developmental of implants that expose function–structure conjunction comparable to inherant cartilage tissue (62). Even though thoroughly study in cartilage engineering, generally, there was no concurrence to type regard, stage and time of mechanical stimulant and growth factor application optimization, or therefore the consolidation of these stimuli for the development of flourishing hyaline-like cartilage. The treatment array capabilities complicate and thereby increase the research cost in cartilage regeneration.

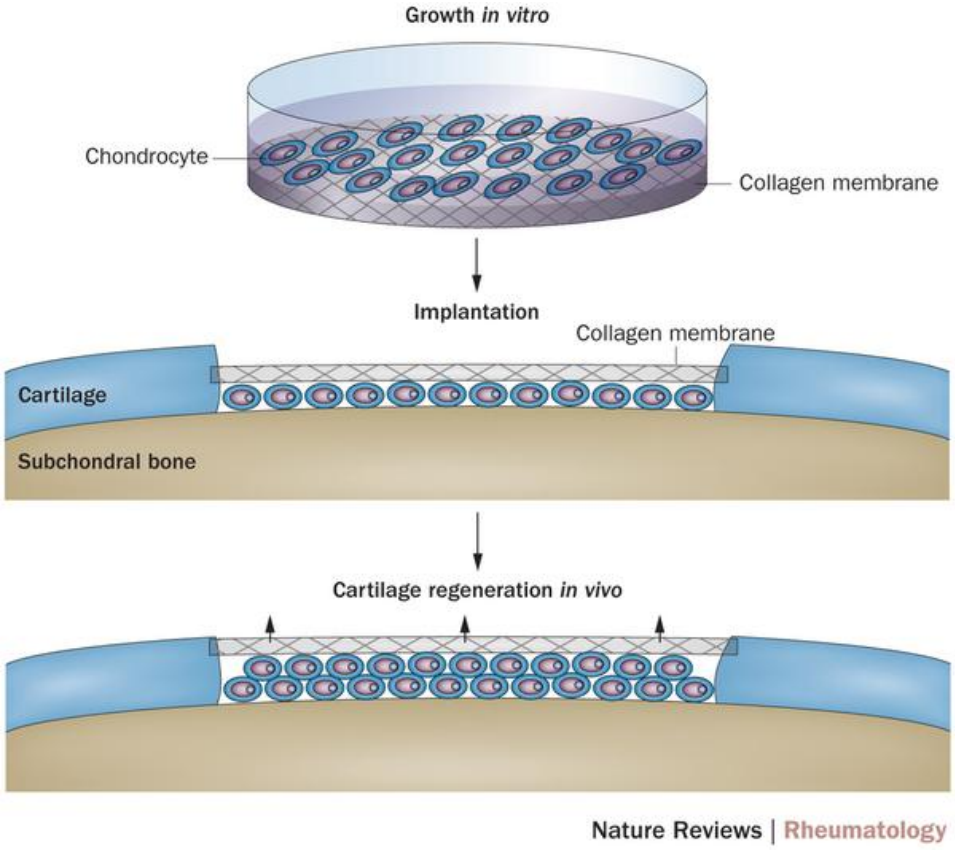


Figure 6. MACI uses chondrocytes that have been pre-seeded into a collagen scaffold and cultured for a period of time before surgical implantation(57) .

The results of autogenic cells-seeded-scaffold-based tissue engineering procedures to regeneration of articular cartilage are applicable in some clinical trials. In phase I clinical of FDA approved trial, eight patients were treated with collagen type I tissue-engineered scaffolds seeded with autologous-chondrocyte, which were administrated with hydrostatic pressure prior to surgery (63)., Cartilage lesions in seven of eight cases were filled relatively entirely by a well-organized neo-tissue after 1 year. The assuring outcomes accompanying to a clinical trial phase II, revealed that after 2 years implantation the tissue engineering-based technique shown the superior clinical results in almost criteria of knee treatment scores and shown a comparable to microfracture safety analysis (64). Although the collagen scaffolds were not possess immunological reactions, however, this potential.use should be evaluated by long-term study

Only autologous chondrocytes was currently utilized as clinically cells-based operations for degeneration treatment of the lesions of articular cartilage. Despite achievement to epitomize the environment within 3D *in vivo* culture, bioreactors, and exogenic biochemical and mechanical stimulant, native hyaline cartilage properties were not yet produced within a repair tissue. Thus, in an achievement for avoidance of some adverse event association with autogenic chondrocytic treatment, the stem cells therapy was developed, abounding of that are recently in an order to approve by FDA for their clinical trialls.

Regarding to the several types of human stem cell, bone marrow-derived MSCs have extensively benefit for clinical application. These MSCs are relatively to harvest and expand easily, acceptance to promise for obtaining and storage for ready to access cell bankings. Additionally, comprehensive *in vitro* and *in vivo* pre-clinical study accepted by FDA demonstrated that MSCs from bone marrow are accomplished to differentiate into chondral tissue (65) (66) and could be use for the regeneration of osteochondrogenic and chondrogenic

tissue. Particularly,in patients with progressive osteoarthritis, bone marrow-derived MSCs them were expressed extracellular proteoglycan and type II collagen and shown a chondrogenic phenotypic maintenance in a matrix in the existence of TGF-β1(67). Another advantageous of MSCs are their migratory capability to defected sites and target tissue, where and are capable for the secretion of T-cell-suppressant cytokines (68) acknowlwdge to be crucial for allogeneic transplantation and also xeno-transplantation. In the case of cartilage restoration, These MSCs could capacitate a repairing system target to promote trophic sense as the result of the releasing of constructed, proliferated and regenerated cytokines exactly to cartilage defects.(69) Aside of the creation a regenerated microenvironment along the deliverance of chemoattractants,(70) These MSCs might also expedite the inherent MSCs migrate to the chondral lesion and support in the regeneration of injured cartilage.

The injectable of bone marrow-derived MSCs for cartilage regeneration have preliminary investigated in human clinical studies one their potential use (Figure 7). This case, four osteoarthritis patients were received each treatment with a single injection of these MSCs into their knee; the result demonstrated that all cases shown the improvement on pain and joint movement with no drawbacks after 1 year.(71) At a 6-month follow-up, acceptable functional and clinical outcomes were also demonstrated in a collateral studied.(72) however the therapeutic utilization of MSCs from bone marrow, there are a lot of resides to be observed: such as, the requirement of MSCs number for promoting chondrogenic restoration; either one or more time treatment requirement to extent the aspiring result; the injection time-window optimization; and the technical safety in long-period. Finally, long-term follow-up prospective controlled studies with was required to investigate for the curative benefit of approved cartilage restoration procedures againt bone marrow-derived MSC injection.

MACI-like and ACI-like techniques can use scaffolds in combination with these MSCs, as againt to patient’s own cartilage cells (Figure 7). A lot of clinical studies were used

pre-cultured MSCs from bone marrow laden in collagen matrices for treating the indicative articular lesions.(74) Reamarkably all researches, which were investigated with limited patient case number, revealed acceptable outcomes after implantation lower 1 year. In an observation of large-scale cohort study , 72 cases with cartilage defects were received the treatment of either implantation of ACI or MSC followed through the concealing with a periosteal flap(75). After 2 year postoperation, The stem cells were found effectively for the treatment of cartilage lesions resemble to ACI in the aspects of clinical outcomes and functionality. Dominantly, the pricing of treatment by MSC from bone marrow was lower than ACI, representing donor site morbidity avoidance and have no requirement of the further operation for obtaining the MSC. Despite, there was incision painful from the site of MSCs were isolated, together with other possible unfavorable events of the added surgery, thus the further investigation should concern on their merit. defect, I/III collagen type, hyaluronan or fibrin scaffold was glued or sutured. d) MACI uses matrices plus either bone-marrow-derived MSCs or primary articular chondrocytes (73)

The affirmative results of these investigations accentuating the possibility of MSC from bone marrow therapy for cartilage defects. Despite, it continues how to be anticipated the bone marrow derived MSCs therapy in the comparison with most approved techniques in clinical investigations comparatively, and additional study was needed to understanding the usefulness of MSC therapy. The possibility account of pre-implantation chondrogenic differentiated MSCs together with biomechanical or biophysical stimulations or growth factors which can advocate phenotype changing and adherance also assurance further study. Since the multipotency properties of MSCs, the confirmatory studies for the safety of MSCs transplantation were required. Finally, additional investigation could assimilate MSCs from bone marrow directly to other MSCs sources on the capability for regeneration of cartilage.

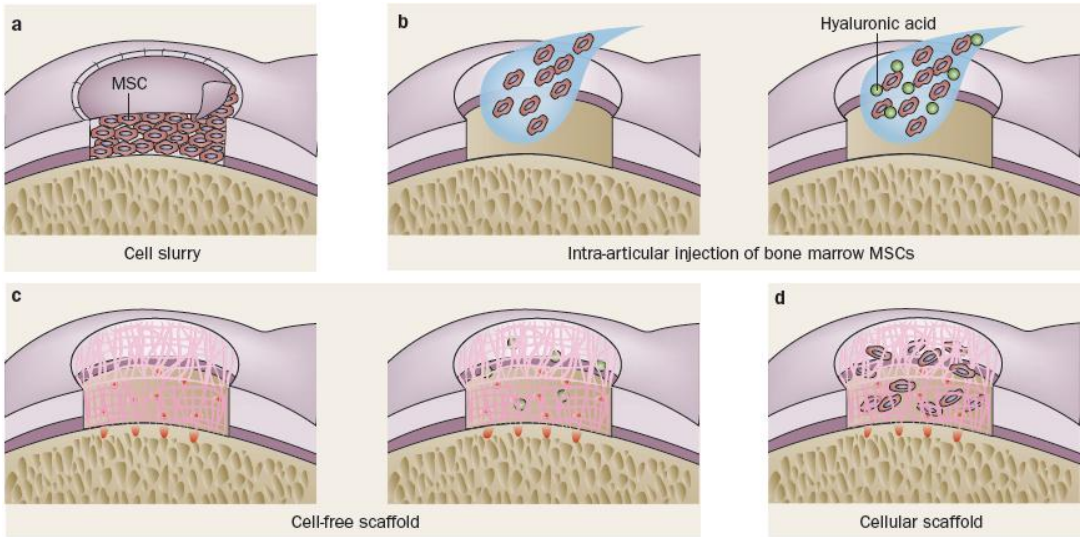


Figure 7. Tissue engineering approches for treating cartilage defects. a) ACI mimicked technique, after debridment the defect is repleted with bone marrow or chondrogesis induced MSCs, and closed with a membrane.from type I/III collagen b) Intra-articular injection of MSCs with or without injectable matrices is a single-stage procedure. c) AMIC is a cell-free, scaffold-based single surgery incorporated with microfracture technique, thereafter in the.

Bone-marrow-derived cells MSCs have been most using in cartilage regeneration study. Although the establishment of bone marrow aspiration is a painful procedure. Despite, MSCs could be obtained from alternative tissues, including fat mass, umbilical cord, muscle, synovial fluid and also periosteum(76). Stem cells from human adipose tissue were the greatest promptly available, and experimental investigation demonstrated adipose-derived MSCs representing a cell growth character and differentiative ability comparable to MSCs from bone marrow(77). Due to the ability for isolation of MSCs in comparatively large quantities, adipose-derived MSCs was identified as excellent MSCs along with the non-immunogenic and anti-inflammatory properties (78) and also minimal ethical deliberation association with their usage compared to MSCs from other sources. However, their proliferation capability and differentiation is deficient likely to be altered by aging of patient. The clinical usefulness and safety of MSCs from human adipose tissue for treatment of degenerated cartilaginous pathology was examined in a patient studies with the injection of these MSCs into synovial cavity of old-age osteoarthritis cases (mean 70.3 years, range 65–80 years) in consolidation by arthroscopy flushing (79). This procedure shown the improvement of physiological character, joint repair and depression of pain; however, this procedure shown the limitation as well as MSC from bone marraow therapy, including the desirable amount of requested cell for promoting chondrogenesis; in one or additional injection requests to accomplish the desirable result; the optimum period for MSCs treatments; and safeness of this procedure. Additionally, quantitative affirmation for the improvement, for example with MRI or biopsy, is required.

Mesenchymal stem cells from alternative sources has also been investigated for chondrogenesis potential; autogeneic peripheral blood stem cells (PBSCs) was extensively studied in clinic, although primarily in case studies (80). A randomized control trial investigated the regeneration of articular cartilage in individuals range from 18 to 50 years old with cartilage defects of knee joint (grade 3 and grade 4 ICRS score) cured with subchondral

drilling arthroscopy subsequented with postoperative introduction of hyaluronic acid intra-articulary, either PBSCs or vehicle alone. The examination revealed that patient treated with PBSC shown the improvement of cartilaginous quality over those of the control group after 18 months of injection which confirm by the ICRS score from visual histological assessment and an morphological MRI scale (81). Additionally, MSCs from synovial fluid represented a superior outcome on chondrogenesis over MSCs from others *in vitro* (82), as shown by hyaline formation of neocartilage development when loaded on scaffolds (83), Despite the fact that the investigations using synovial-derived MSCs were unexecuted. Multipotency dermal cells were harvested and show excessive ability *in vitro* chondrogenic differentiation of study of cartilage tissue engineering (84). *In vitro* study should continue to understand the chondrogenic capability of dermal MSCs and therefore the optimization of chondrogenic differentiation. Prospective pre-clinical and provisional clinical investigation will confidently disclose the regarding problems, account, clinical relevence and profitable of dermal MSCs and then supporting to the explanasion identification and usage criteria's of cartilaginous repair.

Apart from cell-based techniques, freshly osteochondral allografts, resemble to particulated allografts of cartilage, were used in cartilage lesions filling and repairing process promotion (Figure 8). Using allograft tissues are not FDA approved on the advantage, and therefore circumvent expensive and time-consumable studies. The positive clinical benefits of using fresh osteochondral allograft transplantation were observed in active teen to adult populations. These observes have demonstrated that osteochondral allografts were lasted for 1 to 25 years, subject to viability of grafted cartilage cells and mechanic constancy in interface between host and graft; when such criteria were accost, hyaline repair tissue were consistently detected by histological evaluation (85). On the other hand, these grafts are generated by the combination of particulated allograft by a fibrin glue for the construction is later deposited into lesion. Restricted autodigestive property of the ECM dispensates the superficial cartilage,

which the additional of the produced ECM which combine the particulated cartilage tissue for the lesional filling. The acquaintance with osteochondral allografts is retricted to in 2–year follow-up studies, which are almost case reports and patient studies showing possibility, capability and safety, with amelioration in knee scores of subjected patient and imaging demonstration of deisrable lesion replenishment (86). The alternate advent for the introduction of fragments of patient's own particulated into the chondral lesion applies a matrix-based approach. Especially, hyaline tissue is arthroscopic isolated from a less mechanical-bearing area of the knee in a single surgery and then regularly ground and adhered before transplantation onto an absorbable synthesis matrix and placed inside the cartilage lesion. In a anticipated study of clinical safeness, the new scaffold-based allograft with autologous cell technique are comparable to microfracture. The clinical results and images analysis revealed that the scaffold-based procedure showed the superior biophysical results , development of tissue and lesser drawbacks (87). The bigger prospective controlled trial was investigated whether on the mechanical stability of formed neotissue of this procedure, by quantitative MRI analyzes and secondary arthroscopic trial was cancelled by the promotor since the laboriously adherance to the restriction of FDA comprisal criteria. Further investigations might compare the changing of structure (as determined by quantitative MRI or authethicated procedures for joint morphology) with either clinical-based evaluation score or case- result stated evaluation methods.

Despite MSC-based and autologous chondrocytes procedures propose an adequete affiance for regeneration of articular cartilage, harvesting tools, cells growth and differentiating are time consumable and expensive. In addition, because hyaluronan matrix and MACI are cellular interventions with scaffolding technology, the FDA acknowledged them as bio-medical devices, appearing in long-term and costly approval regulation. The regulatory benefits through the attraction enhancement in the cellular free procedure for chondra defect repair and regeneration.

The cellular-free method called autologous matrix-induced chondrogenesis (AMIC, Figure 7c) can accomplish in a onetime of surgical operation. In AMIC, a minimize arthrotomice cleans and exposes the lesion area, MSCs in mesenchymal and marrow mixture through were released by microfracture, and finally the composite of type I and III collagen scaffold is then fixed into the lesion area (88). The inserted scaffold is stabilized by the resulting of clot blood which help the promotion of primitive mechanic stabilization and chondral repair. Patient studies demonstrated that AMIC was shown the safety and efficacy for treatment distinct-deepness cartilage lesions (89, 90).



Figure 8. Neotissue formation by the combinantion of native particulated cartilage with fibrin glue. autodigestion of ECM releases was limited to superficial chondrocytes, which additional ECM was generated that integrates the cartilage pellicles and filling the lesion (91).

In a report of the 5 years followed-up for study shown abundantly improve of Cincinatti, ICRS, Lysholm and Tegner scales after surgical operation in 1 years (92). In addition, Radiological Imaging resulted middle-to-full stuffing of cartilage lesions. The some other AMIC investigation for osteochondral defect comprising of poly(lactic-*co*-glycolic acid) in the cartilaginous area and biphasic calcium triphosphate biodegradable scaffold in the ossified area was utilized for treatment cartilage lesions of talus (93). At a year of follow-up, American Orthopaedic Foot & Ankle Society and Ankle Osteoarthritis Scale clinical scores were elevated in six patients resembled to the prior to operative rate. Arrestingly, the observation of clinical results in AMIC-cured patient's were not associate with aging, body mass index or the previous orthopedic surgeries time of the cases. The concise follow-up of small number of cases and not having controlled cases might account for the deficient association. The directness and economical of AMIC benefits the additional investigation for identification on the aspect of neo-cartilage generation and reproducible information and any potentiality difficulty association with AMIC.

A novel approach for the enhancement of cartilage repair by AMIC is the delivery of growth factors which the specific recruitment and stimulation of stem cells from the subchondral tissue into cellular-less matrices. Specific cytokines can be tailor-made for the activation of chondrocytes surrounded the healthy tissue for helping the remodeling, repairing and also tissue filling of the cartilage lesion.

Implantation of polymer-based materials next to the early microfracture of a lesion in combination with patients serum or PRP and hyaluronan was considered as a new approach for AMIC *in situ* activation (94). Platelet-rich plasma and autologous serum could augment MSCs from bone marrow (95), and hyaluronan shold assist the chondrogenic differentiation of MSCs from bone marrow (96). A clinical trial revealed that 52 patients receiving this treatment showed the improvement of Knee injury and Osteoarthritis Outcome Scores

(KOOS) at 1 year later to the treatment, resembled to histological analysis demonstrated that lesions of patients were stuffed with a cartilage-like restoration (97). However, controlled trials with a long-perid observation are required for the confirmation of information and affirm that the neocartilage is long-lasting. An alternative technique, chitosan in a glycero-phosphate solution is combined with patient whole blood for a freshly clot forming. The comparatively of multiple site randomized clinical study at 12 mount later to surgery operation demonstrated that the combination treated with chitosan–glycerol-phosphate and microfracture resulting the better filled defect and a better qualify of restoration over microfracture apart (98). However, no significantly different of functional detected outcomes between AMIC and microfracture. Likely, the formation fibrocartilage later on microfracture and started the deterioration with 24 mounts after surgery, 4 year long-period studies for their function.

Proteins, serum from periphery, chemoattractant and PRP can use a component of AMIC for *in situ* activation (99). Conceivably, these regimens could be tailor-made for a condition and anatomical location specifically. Despite almost regimens was approved by FDA and was directly applicable to clinic The controlled trials followed by adequetely-devise pre-clinical investigation are initially required for the investigation on the forcefulness. Furthermore, TGF-β superfamily and their affiliated growth factors along with insulin-like growth factors, PDGF and pro-epidermal cytokine, can induce MSCs to migrate. Therefore procedures were created for the combination of cytokines for cell growth and MSC chemotatic factors for the additional promotion of chondral restoration by accommodating the releasing by disciplined aspect.

Although either cells or exogenous stimulation was used in several tissue engineering approaches for cartilage regeneration, alternative choice is a auto-assembly processing or scaffold-less based approach (Figure 9) (28). Scaffold-less technology can made an

interruption of cell to cell signaling and force protection, the microenvironment of neocartilage increased in their bioactivity, reaction to stimulants enhancement and can integrate to surrounded area. *Ex vivo* studies shown the invigoration of self-assembly chondral tissue with exogenic factors (hypoxia, growth factors, cross-linking agents) and mechanical (hydrostatic pressure) stimuli can promote the generation of healthy neocartilage comparable to native cartilage functional properties (100, 101).These researchers also demonstrated the scaffold-null neocartilage represent in both *in vivo* maturation and integration capabilities (102). In comparison with scaffolding-based approaches, the neocartilage from scaffoldless technique shown a higher percentage of ECM and also better in mechanical properties. Thus, scaffold-free neotissue is hold promise for novel development for the development of awfully bio-implants for chondral restoration.

The generation of spheroids for autologous chondrocytes (chondrospheres) implantation is alternative scaffoldless method for chondral restoration. In a degenerative experiment by minipig, the chondrospheres shown the adherance and integration with full-depth chondral lesions and chondrogenic ECM production (103). The competency of chondrospheres in clinic is recently studied in clinical trial at phase III in Europe; the primarily results in clinic were not reported. The utilization of juvenile allogeneic chondrocytes is also another scaffold-free technology. Experimental studies have shown that chondrospheres are more strength and more stable in their chondrogenic property over mature cells and can bypass immune reaction and chondral tissue was formed after transplanted into goat chodral lesions(104)..

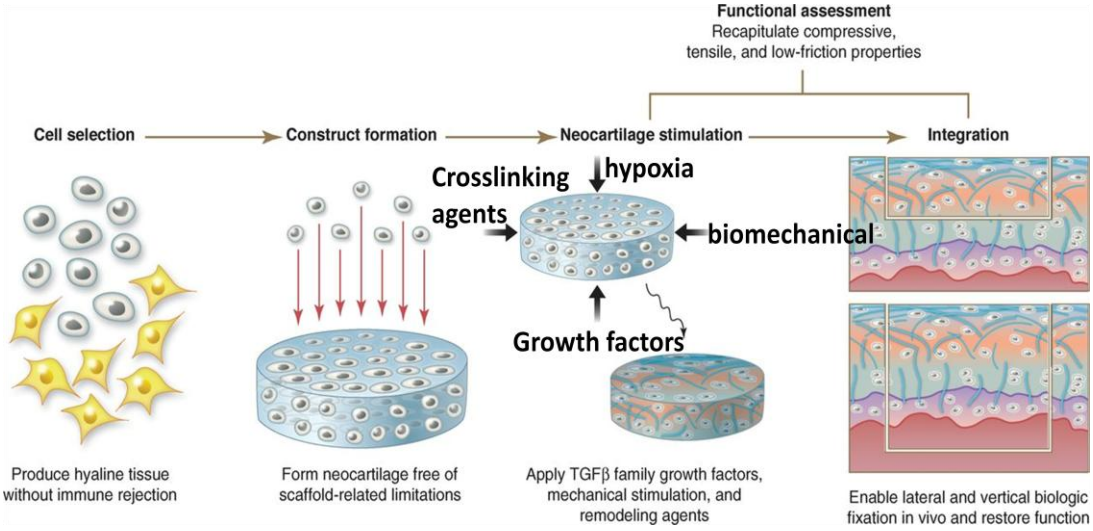


Figure 9. Scaffoldless tissue engineering. The cell source chosen for cartilage generation, treated with appropriate culture conditions, must have the ability to produce a matrix specific to articular cartilage and must not evoke an immune response. TGF-β family growth factors, physiologic mechanical stimulation, and matrix-remodeling enzymes have shown a large degree of promise as stimuli (28).

2.4. Silk fibroin for tissue engineering

Silk fibroin is a natural protein synthesized by the silkworm. In general silkworm is devided into two groups i.e., mulberry or domesticated silkworm as well known as *Bombyx mori* and non-mulberry or wide silk (105). Silk fiber from cocoon of *B. mori* contains at least two major fibroin proteins, light and heavy chains, 25 and 325 kDa (106), respectively. Its major amino acid component comprises of glycine, alanine, and serine. The protein can be prepared to form a variety of new materials(107) i.e., films, nanofibers, scaffolds, membranes, hydrogels, and powders, which renders it extremely suitable for the application in a large variety of use in the field of biomaterials, tissue engineering and drug delivery (Table1).

In terms of cell compatibility, degummed silks are arguably well suited for cell culture purposes. SF coated membranes (prepared using aqueous fibroin solutions) permitted greater adherence and consequently greater proliferation of fibroblasts in comparison to non-coated membranes (108). Silk fibroin films from regenerated *B. mori* fibroin has also been shown to be biocompatible to human mesenchymal stem cells *in vitro*, and even enhanced cell proliferation compared to collagen scaffolds. *In vivo*, inflammatory reactions to the silk films were either similar to or less than to collagen(109).

Table 1. Composition and biophysical properties of silk derived from *Bombyx mori*

	Bombyx mori
Major structural protein	**Fibroin**
Main component of protein	**Heavy-chain fibroin 5623 aâ (110)** **Light chain fibroin 262 aa (111)**
Coating of thread	**Sericin, antigenic proteins (21)**
percentage of crystallinity	**36-66 (112)**
Major amino acid	**Gly (46%), Ala (29%) and Ser (12%) (112)**
Repeating motifs	**GAGAGS and GAGAGY [(113)]**
Content of crystalline (hydrophobic) regions	**Mostly the repeating motifs (112)**
Content of non-crystalline (less hydrophobic) regions	**Negatively charged, polar, bulky hydrophobic and aromatic residues(112)**
Property	**Ref (106) and (112)**
Density in g/cm^3	**1.35-1.42**
Crystallite size in nm	**1.0-2.5**
Index of refraction	**1.591 parallel to fiber**
Maximum use temperature in °C	**170**
Thermal degradation in °C	**250**
Heat capacity in j/g K	**1.38**
Glass transition temperature	**178°C at 0%RH**
Mechanical properties	
UTS (MPa)	**740**
Modulus (GPa)	**10**
% Strain at break	**20**
Biodegradation	**> 60 *in vivo*** **Fibers lose most of their tensile strength within one year in vivo,**
Behaviour in water	**Little change in silk shape/length(less hydrophilic)**

Table 2. Biomedical Applications of Fibroin-Based Materials (107)

Type of polymer	application
nonmulberry and mulberry silk gland fibroin	**adipose tissue**
fibroin, collagen/fibroin, polylactide/silk fibroin-gelatin, fibroin modified-polyhydroxyalkanoate	**blood vessel**
silk fibroin/chitosan/PLLA, chitosan/fibroin-hydroxyapatite, nonmulberry silk gland fibroin, nonmulberry and mulberry silk gland fibroin	**bone**
silk fibroin modified porous poly(e-caprolactone), plasma-treated fibroin	**cartilage**
alginate/fibroin, silk fibroin/gelatin	**drug delivery**
gelatin/silk fibroin, hyaluronan/silk fibroin, chitosan/silk fibroin, fibroin/recombinant human-like collagen, antheraea assama silk fibroin, nanohydroxyapatite/fibroin, silk fibroin-modified PHBHHx, polylactide/silk fibroin-gelatin	**general**
gelatin/silk fibroin	**ligament**
fibroin/recombinant human-like collagen, PLLA/fibroin, chitosan/silk fibroin, chitosan/silk fibroin/heparin	**liver**
***Antheraea pernyi* silk fibroin**	**tendon**

2.5. Biomedical Application of hyaluronic and their derivative

Hyaluronic Acid (HA), also known as hyaluronan, is a nonsulphated glycosaminoglycan, composed of alternating units of D-glucuronic acid and D-N-acetylglucosamine, linked together via alternating β-1,4 and β-1,3 glycosidic bonds (Figure 10) (114). HA is a ubiquitous component of the extracellular matrix of skin, cartilage, intervertebral disc, vitreous body of eye and synovial fluid. HA has been purified with varying molecular mass from joint fluid, umbilical cord, rooster comb and certain strains of streptococci (115) . The biophysical properties of HA such as high viscosity, elasticity and highly negative charge make HA useful in various therapeutic procedures. HA has been widely used in the cosmetic, nutritional, pharmaceutical and medical fields.

HA has been recognized as “bio-lubricant” and “bio-polymer filler”. HA plays an important role in embryonic development, tissue organization, wound healing, angiogenesis, cell migration, extracellular matrix formation and tumor invasion (24). HA also possesses anti-inflammatory activity and is under evaluation to be used as a therapeutic agent for a number of inflammatory disorders (116). The unique biophysical characteristics, biocompatibility, non-immunogenic properties and ease of size chain manipulation make HA a useful biomaterial for scaffold and carrier design in tissue engineering. HA has been manufactured in a variety of physical forms including hydrogels, sponges, meshes, fibers and fabrics that allow the development of a variety of HA-based scaffolds. More importantly, HA can be used as cell carrier in tissue engineering and regenerative medicine (117). HA can be created as a chemically cross-linked hydrogels and have been used as a depot system for controlled gene or DNA and drug delivery for tissue regeneration (118). In addition to application for biomolecules and drug delivery, HA based scaffolds have the potential to be used as a cell delivery system (119). In the 1970s, the first hyaluronan-based biomedical product (Healon) was developed and approved by FDA for the use in eye surgery (e.g.,

corneal transplantation) (24). Recently, the commercially available HA-based product, Hyaff (i.e., benzyl ester of HA) is the most often used in clinic (120). In Table 3, an overview is given of various of the derivatives hyaluronic acid and their corresponding biomedical applications.

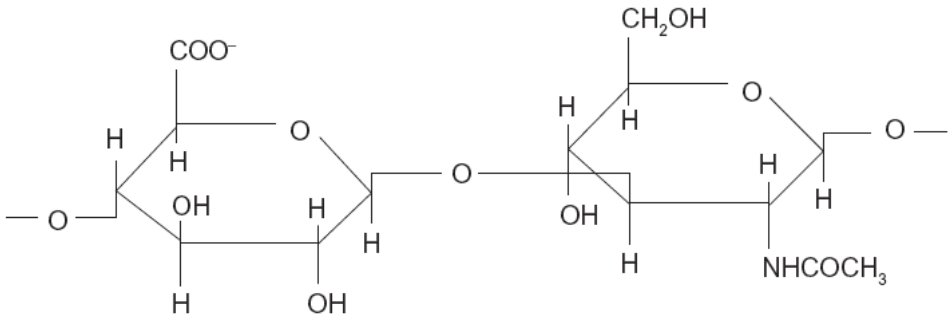


Figure 10. Structure of the repeating disaccharide unit of hyaluronan.

Table 3. Biomedical Applications of Hyaluronic Acid (107)

Type of hyaluronic acid	**Application**
ester-containing hyaluronic acid	adipose tissue
amine/aldehyde-containing hyaluronic acid, hyaluronic acid/poly(vinylalcohol), MMP-sensitive hyaluronic acid	bone
hyaluronic acid/collagen I, hyaluronan/gelatin/chondoitin-6-sulfate, adipic dihydrazide-modified collagen/hyaluronic acid, fibrin/hyaluronic acid, chitosan/hyaluronic acid, chitosan/hyaluronic acid, carrageenan/fibrin/hyaluronic acid	cartilage
acryl-modified hyaluronic acid/poly(ethylene glycol) acryl	gene therapy
ester-containing hyaluronan/butyric and retinoic acid, methacrylated hyaluronan, divinylsulfone cross-linked hyaluronan	heart
benzyl esters of hyaluronic acid, hyaluronan/gelatin/chondoitin-6-sulfate	intervertebral disk
benzyl esters of hyaluronic acid	liver
hyaluronic acid	muscle
photo-cross-linked hyaluronic acid, collagen/hyaluronic acid, fibroin/hyaluronic acid, antibody-modified hyaluronic acid, hyaluronic acid/polylysine	nerve
hyaluronic acid derivatives, carbodiimide-cross-linked hyaluronic acid	ophthalmology
benzyl esters of hyaluronic acid, hyaluronan-gelatin, EDC cross-linked hyaluronan/chondoitin-6-sulfate/gelatin, adipic dihydrazide derivatives of hyaluronic acid/PEG-propiondialdehyde, hyaluronic acid/chitosan/gelatin	skin
thiol-modified hyaluronic acid	spinal cord
methacrylated hyaluronic acid	vascular tissue

2.7. Hyaluronic acid-based for cartilage tissue engineering

The regeneration and repair of articular cartilage is a complicated due to there is avascularized and scarely cellularized tissue. Thus, only surgical technique can be used for the repairing of the injured articular cartilage. This ultimately brought about the development of cartilage engineering and cellular therapy approach such as synthetic ECMs for the replacement of injury. Synthetic ECMs are comprised of ECM-derived protein and artificially sulfated glycosaminoglycans (GAGs), specifically HA. The sulfated GAGs takings component for cell signal pathway in which adhere to cytokines , chemokines and growth factors, and therefore involve in regulation of restoration. Sulfated HA polymer laminating is crucial for the enhancement of the biocompatibility property of organic/inorganic composites. Sulfated HA plays as potent stimulant of anaerobic action of tendon , cartilage, bone and also spine, and therefor MSCs recruitment to the injured tissue, and promotion target cell differentiate. Apprehension of the possibility of sulfated-HA therapy to the responsibility for targeting cell is a field of highly concerning (121). This point, collagen/HA scaffold was produced by modified HA molecules with cross-linking to formyl group with collagen, demonstrated the superior osteoconductivity and elevated biocompatibility. HA-gelatin hydrogels generated by disulfide cross-linking was competently mimicking ECM due to their enzymatic degradation resistant and biocompatibility therefore fibroblasts adherence and proliferative promotion (122). Additionally, the N-succinyl-chitosan-aldehyde HA composite hydrogel contributed for encapsulation and attachment of chondrocytes (123), indicated that these material was potentially employment in cartilage engineering. HA hydrogels were exploited for the development of chondral restoration with MSCs therapy. Human embryonic stem cells (hESCs) needed of any undifferentiated teratogenicity was utilized in cartilage repair and regeneration as they contribute an endless derivation of chondrocytes in the presenting of particular cytokine. Furthermore, the HA hydrogel for repairing of cartilage lesion of rats using chondrocyts acquired from hESC demonstrated an assemble of

cartilaginous-rich of neotissue (124). HA-based scaffolds (e.g. HYAFF®11) seeded with bone marrow concentrate (BMC) advocated the chondrogenesis of stem cells. BMC can play as MSCs displacement (125). HA-Collagen scaffold display pore reduction in sizes and MSC filter through elevation together with the expression augmentation of collagen type-2 and SOX-9 and elevated chondral ECM production. Hence, the applications of HA-collagen scaffold were extended in chondral regeneration (126). In the comparison with alternative synthetic ECM development, a human mesenchymal stromal cell (hMStC) cultured in synthetic ECM comprising HA sulfates and collagen demonstrate the elevation of the displacement of calcium phosphate and of non-specific alkaline phosphatase activity. The HA sulfates-collagen comprising synthetic ECM was not alter the expression of *runt*-related transcription factor-2 and osteocalcin (osteogenesis markers), while the expression of bone sialoprotein II was attenuated. The outcomes suggested that artificial ECMs containing HA-Sulfates-collagen are capable for the activation of osteogenic differentiation of hMStC , that made these scaffolds valuable for tissue engineering by hMStC and developmental of biomaterial for promoting formation of bone (127). The hydrogel An oxidized constructed resveratrol and HA achieve the collagen type-II, MMPs and aggrecan genes expression and also reduced the chondral inflammation by lip-polysaccharide-induced. The characters of these hydrogel make them was applicable for chondrocyte carrier for the inflammation blocking (128). 3Ds scaffolds were also to be adorable biomaterials for chondral regeneration and cartilage tissue engineering. HA mixed with silk fibroin to fabricated of sponges demonstrated the improvement of the chondrogenic charcteristic (129). Currently, a simuteneous repair activity of chondral lesions in raprine model with polycaprolactone scaffolds modified with HA implantation was reported (130) . Absolutely, these investigation indicated that hydrogels from HA with or without additives could be used for the successfully cartilage healing.

Chapter 3

Materials and Method

3. Materials and Methods

3.1. Preparation of regenerated silk fibroin

Silk cocoons from *Bombyx mori* were kindly provided by Queen Sirikit Sericulture Center, Narathiwas, Thailand. Regenerated silk fibroin solution was prepared as previously described (131). Briefly in the degumming process, raw silk cocoons were boiled twicely in 0.25% (w/v) Na_2CO_3 (Merck or Sigma, 1g degummed silk/100 mL) for 30 min in order to remove sericin, waxes and other impurities and then washed several times with deionized water. The air-dried degummed silk fibroin fibers were dissolved in 9.3M LiBr (Merck 2g/10 mL) for 4 h at 60°C (figure 11). The solution was centrifuged at 4000rpm for 10 min to exclude the insoluble residue and then dialyzed against deionized distilled water by using dialysis membrane (MW3500, SpectraPor, USA) for 72 h. The concentration of the obtained silk fibroin was determined by Biuret assay and then adjusted to 6% w/v.

3.2. Preparation of blended SF/HA scaffolds

HA was weighed according to the weight required for fabrication by the silk fibroin to HA ratios (w/w) of 100:0 (SF100), 90:10 (SF90), 80:20 (SF80) and 70:30 (SF70) and then dissolved in the purified SF solution (figure 11). The blends were incubated under slight agitation with a magnetic stirrer for 1 h at room temperature in order to complete the dissolution of HA and to allow possible interactions between the two components. The total solid weight of SF and HA in the mixtures were controlled at 3% w/v. After completely mixing, the SF/HA blends were then reacted with EDC/NHS (5%w/w) for 15 min under mild

agitation. Then the mixtures were left for 2 h at room temperature to allow the SF-HA to crosslink. Finally, 2 mL of crosslinked solution was added in each well of 24- wells polystyrene tissue culture plate (TCPS) and frozen at -20°C overnight prior to lyophilize at -80°C for 24 h. Additionally, the freeze-dried scaffolds were treated with 80% methanol beneficial to stabilize the scaffolds in aqueous solution (132) and also sterilize the scaffolds.

3.3. Pore size mesurement

The pore size of scaffolds each group was analyzed from SEM image of freeze-dry scaffold by the ImageJ software (v. 1.48). The pore size of the scaffolds were randomized sampling (n=20) to calculate the average pore size.

3.4. Microarchitecture observation, mechanical and physical properties of SF/HA scaffolds

Scaffolds morphology and pore sizes were observed by scanning electron microscopy (SEM-JSM5800LV, JEOL, USA) at an operating voltage of 20 kV was used for scaffold imaging. In order to observe their structure, the scaffolds were broken vertically in liquid nitrogen and sputtering with coated gold prior to investigate by SEM.

The swelling properties of SF/HA scaffold were also investigated as previously described (133). In brief, the dry weight of scaffolds was measured (W_0) before submerged in PBS for 24 h at room temperature. After removal of excess PBS, the wet weight of the scaffolds (W_t) was measured. The swelling ratio, *S,* were then calculated by using this equation.

$$S = \frac{(w_t - w_o)}{w_0}$$

Where w_o is the initial weight of dried scaffold at time t = 0 and w_t is the weight of hydrated scaffolds at 24 h, t. The values were reported as the mean ± standard deviation (n=6)

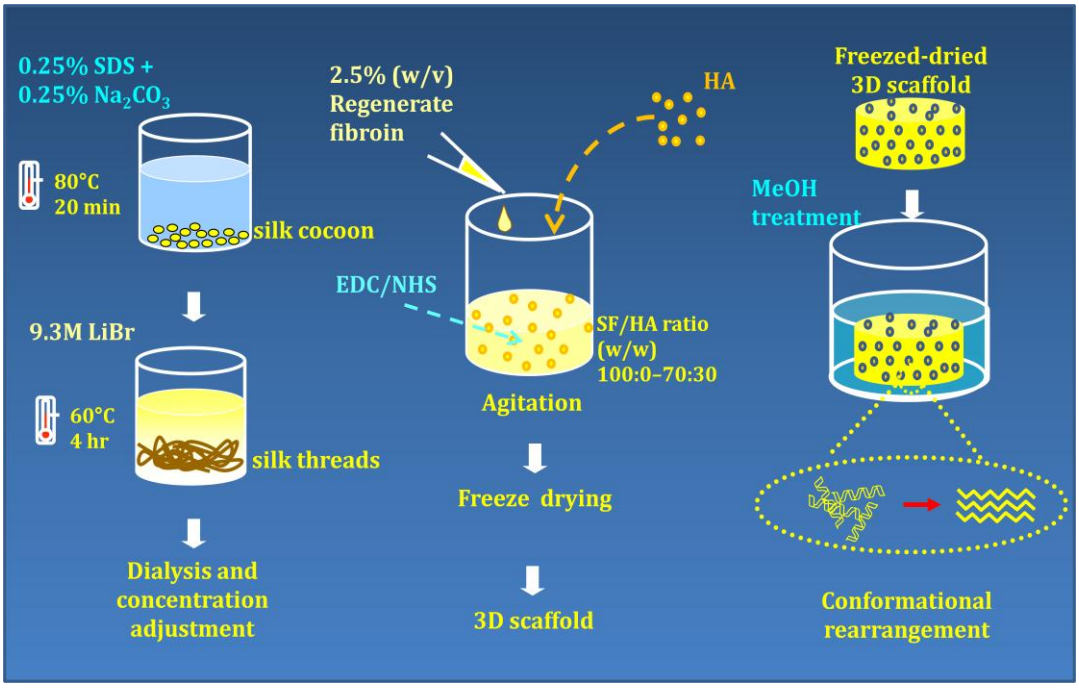


Figure 11. Regenerated fibroin preparation and fabrication of SF/HA scaffolds.

The cylinder shape scaffolds (n = 6) were also subjected to unconfined compression tests by using the Universal Testing Machine (Lloyd instruments, LRX-Plus, AMETEK Lloyd Instrument Ltd., Hampshire, UK) which equipped with a 10N capacity load cell. Tests were conducted at room temperature in PBS (wet state) at a constant compression rate of 2 mm min^{-1}. The compressive stress and strain were graphed by NEXYGEN software for the measured sample dimensions: cross-sectional area and sample height (measured automatically at 0.01 N preload). The compressive modulus and standard derivation were analyzed after testing. The compressive moduli were calculated from the slope between 5% and 10% strain of the compressive stress-strain curve. The values were reported as the mean ± standard derivation (n=6).

3.5. Isolation, characterization and differentiation of HUMSCs

Human umbilical cords from both sexes were collected from full-term births after either cesarean section or normal delivery with informed consent which approved by the Prince of Songkla University Ethics Committee for Human Research at the Songklanakarind Hospital, Hatyai, Songkla, Thailand (EC.55-037-25-4-3).

The human umbilical cords were processed and the HUMSCs were isolated as previously described (134) (figure 12) with some modifications. Briefly, umbilical cord samples 3–5 cm in length was used for the isolation HUMSCs. Samples were processed within a maximum period of 48 h since their collection. The umbilical cord samples were rinsed in 75% ethanol for 30s and then submerged in PBS. The umbilical cord blood vessels were removed and the gelatinous tissue surrounding the vessels was subsequently cut into very fine pieces about 0.5–1 mm^2. The pieces of umbilical cord tissue were then placed on a sterile 100 × 20 mm petri dish and left for 5–10 min at room temperature, to facilitate tissue attachment. The minced tissue were carefully covered with 5mL of DMEM-LG medium (Gibco®, Life technologies, USA) supplemented with 10% inactivated fetal bovine serum

(Gibco®, Life technologies, USA), 2 mM L-glutamine, 1X of antibiotic and antimycotic (Gibco®, Life technologies, USA). The umbilical samples were incubated at 37°C in a humidified atmosphere of 5% CO_2 incubator for 5–10 d, before visible colonies of WJ HUMSCs were observed. The extruded cells were consequently trysinized (0.25% trysin [Gibco®, Life technologies, USA]) and then transferred to 75-cm^2 flasks at a density of $5 \times 10^3/cm^2$ (135) and cultured up to 70–80% confluency. Cryopreservation was performed when cultures reached 70–80% confluency. In this study HUMSCs was used to investigate further at 3 to 6 passages of monolayer cultured cells.

To determine multipotency phenotype, HUMSCs from passage 2-4 were stained with antibodies against CD10, CD13, CD34 and CD45 (all from BD Biosciences, USA). Mouse isotypic antibodies served as the control. At least 10,000 labelled cells were analyzed by Beckman Coulter Cytomics FC 500 flow cytometer. For osteogenic differentiation, 70–80% confluent cells were incubated in DMEM-f12 medium supplemented with 10% FBS, 2mM L-glutamine (Gibco®, Life technologies, USA), 50 µg/mL of ascorbic acid, 0.1 µM dexamethasone (Sigma), 10 mM β-glycerophsphate (Sigma). The depositions of mineralized calcium phosphate were visualized by alizarin red staining after 21 days of culture. For adipogenic differentiation, the confluent HUMSCs were incubated with StemPro® adipogenesis differentiation kit (Life technologies, USA). At day 14, the lipid droplets was confirmed by oil red O staining (Sigma-Aldrich). For chondrogenic differentiation, the confluent HUMSCs were treated with StemPro® chondrogenesis differentiation kit (Gibco®, Life technologies, USA). At day 14, the differentiated cell were stain Alcian blue 8GX (Sigma) and then visualized.

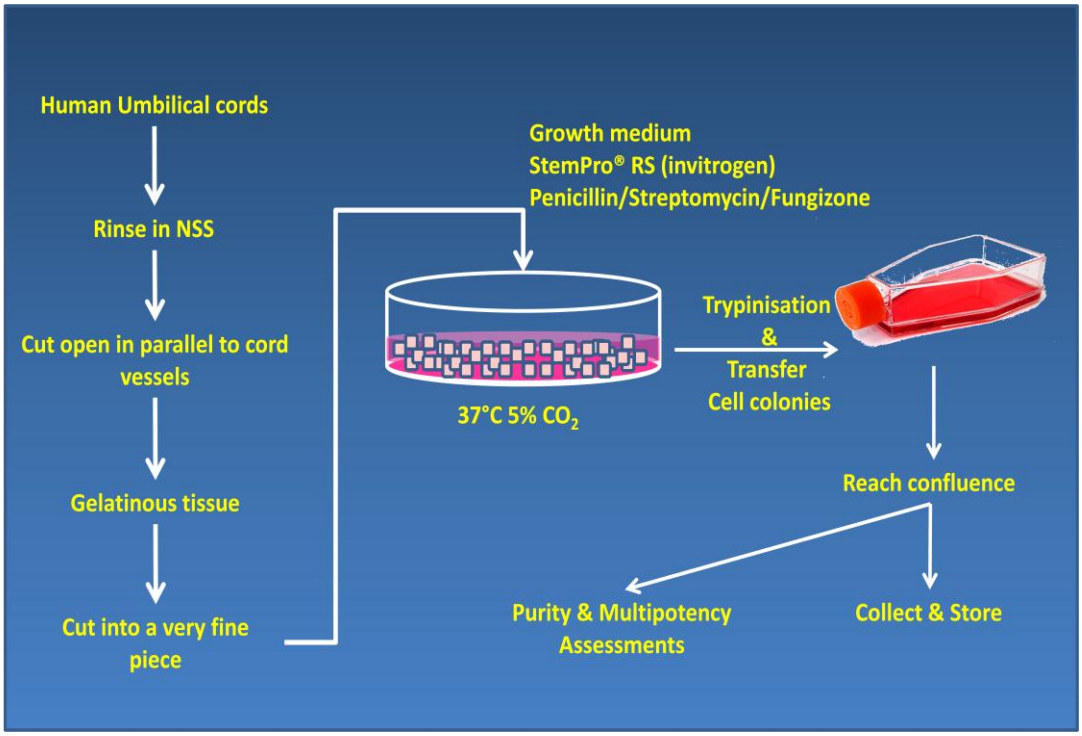


Figure 12. HUMSCs isolation and expansion.

3.6. HUMSCs proliferation and differentiation in Silk/HA scaffolds

For HUMSCs attachment, a freeze-dried (~3 mm thick, ~8 mm diameter) of the different types of silk/HA scaffolds (SF100, SF90, SF80 and SF70 n = 6) were incubated with cell culture medium for HUMSCs isolation (as described) overnight before cell seeding. HUMSCs were seeded in the silk/HA scaffold at a density of $2.5x10^5$ cells/scaffolds and then incubated in 5% CO_2 at 37°C. The proliferation of HUMSCs under chondrogenic condition (discuss further) over the experiment period was evaluated by the utilization of resazurin dye of attached cells.

Chondrogenic differentiation of HUMSCs was performed in both pellet and also 3Ds culture experiments for 4 weeks. In brief, for the pellet culture system(136) the trypsinized HUMSCs were resuspended in chondrogenic medium (StemPro® chondrogenesis differentiation kit) at a density of 5 $x10^5$ cells/mL. Each 500 □l of suspended cells were centrifuged at 600xg for 5 min in 15-mL polypropylene conical tubes. Pelleted cells were incubated at 37 C under 5% CO2 prior to analysis. Chondrogenesis of HUMSCs in pellet culture was evaluated by haematoxylin and eosin (H&E) and alcian blue staining. For the 3Ds culture experiment, HUMSCs were seeded at $1x10^6$ cells per scaffolds. The chondrogenic induction of HUMSCs in 3Ds culture was performed after 7 day of initial seeding of cells into SF/HA scaffolds. Cell-seeded scaffolds were collected at day 7, 14, 21 and 28 after induction. The chondrogenesis was evaluated by using real-time PCR and histochemical and immunohistochemicals analysis.

3.7. Cell viability and fluorescence micrograph

Cell viability of HUMSCs in constructed scaffolds were evaluated by fluorescence-based live-dead assay by using fluorescein diacetate (FDA, Sigma) and propidium iodide (PI, Sigma), which stain viable cells and dead cells, respectively. At day 28 after chondrogenic

induction, scaffold were incubated in DMEM-LG containing 8 µg/mL FDA and 20 µg/mL PI for 5 min at 37°C in 5%CO2 atmosphere. After removal of the staining solution, scaffolds were rinsed with PBS and then incubated in culture medium prior to monitor using fluorescence microscope (Olympus IX71).

Cells morphology in 3Ds matrices were also observed by fluorescence microscopy. Chondrogenic-induced HUMSCs-seeded scaffold were fixed in PBS buffered (pH7.4) containing 3.7% para-formaldehyde for 5 min and then permeabilized the cells using 0.1 % Triton X-100 for 5 min at room temperature. After blocking nonspecific binding with 3% BSA, actin filaments were labeled by incubating cells-seeded scaffolds in 25 µg/ml fluorescein isothiocyanate-conjugated phalloidin (FITC-phalloidin conjugate, Sigma-Aldrich, USA) solution in PBS for 20 min. the nuclei were counterstained with 2.5 □g/mL bisBenzimide H 33342 trihydrochloride for 5 min at room temperature. Fluorescence images of actin filaments and nuclei were acquired by using fluorescence microscope.

3.8. Histochemistry and Immunohistochemistry

On day 28, cell-constructed scaffolds were fixed overnight in 3.7% paraformaldehyde in PBS buffered (pH7.4) and then dehydrated in a gradient series of ethanol, cleared with xylene, and embedded in paraffin blocks. Five-mm of paraffin embedded sections were stained with H&E for the observation of cell morphology in scaffold and alcian blue for the presentation of sulfated glycosaminoglycan (GAGs).

For immunohistochemical analysis, mouse anti-human type II collagen monoclonal antibody and mouse anti-human aggrecan monoclonal antibody (all from EMD Millipore, Temecula, USA) were used as a primary antibody for the evaluation of the expression of collagen II and aggrecan. In brief, the sections from each scaffold were neutralized of their endogenous peroxidase activity by using Novocastra Peroxidase block. Section were blocked

in 0.4% casein/PBS for 5 min at room temperate and the reacted with primary antibodies (dilution 1:250) at 4 °C for 2 hour. After post primary incubation, the immune histological staining was performed by using Novolink™ polymer detection system (Leica Biosystems, Newcastle, UK) following to the manufacturer's protocol.

3.9. RNA isolation, cDNA synthesis and real time PCR

Gene expression profile of HUMSCs constructed scaffolds were analyzed at days 7, 14 and 28 after chondrogenic induction. Constructed scaffolds were homogenized and RNA was extracted from differentiated cells using a TRIzol® reagent (Life Technology, USA) with manufacturing instruction. Total RNA was reverse transcribed to cDNA by using the Superscript® III reverse transcriptase (Invitrogen, Carlsbad, USA). with Oligo$(dT)_{20}$ primers (Invitrogen, Carlsbad, USA). Primers specific to human mRNA for collagen type II (col2a), aggrecan (agg) and Sry-type high mobility group box transcription factor 9 (sox9) are show in Table 4. Quantitative relative expression of each targeted gene was analyzed on a CFX96™ Real-Time PCR detection system (BioRad, USA) with EXPRESS SYBR GreenER qPCR supermix (Invitrogen, Carlsbad, USA). The expression values of targeted genes (n=6) were normalized to the endogenous expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

3.10. Statistical analysis

All values in graphs and tables were presented as the mean ± standard derivation, and each type of experiments was six times replicated for mechanical testing, swelling test and also gene expression analysis and 20 times for the randomizing data of pore size. One-way ANOVA with Turkey's test was used for statistical comparisons. P-values of < 0.05 were considered statistically significant.

Table 4. Primers and their sequences for quantitative real-time PCR analyses.

Gene	Primer-sequence	Product size (bp)	Accession number
GAPDH	Forword 5’-gagtcaaccgatttggtcgt-3’	242	XM_005253678.1
	Reverse 5’-ttgattttggagggatctcg-3’		
Col2a	Forword 5’-accccaatccagcaaacgtt-3’	151	NM_001844.4
	Reverse 5’-atctggacgttggcagtgttg-3’		
Agg	Forword 5’-acagctggggacattagtgg-3’	189	NM_001135.3
	Reverse 5’-gtggaatgcagaggtggttt-3’		
Sox9	Forword 5’-gcggaggaagtcggtgaagaacgggca-3’	786	NM_000346.3
	Reverse 5’-tgtgaccgggtgatcggcggg-3’		

Chapter 4:

Results

4.1. Scaffold morphology

The SEM micrographs of pure silk fibroin and SF/HA scaffolds with different blending ratio are shown in figure 13. As observed, pore size were considerably larger when increase content of hyaluronic acid. In addition, formation of pores in scaffold was highly organized when hyaluronic acid was introduced into scaffolds. The pore thickness was also markedly increased when increase hyaluronic acid content. The surface of pore was considerably smooth in SF 100 and more rough increase hyaluronic acid. Moreover, the fibrous architecture was observed in SF70 whereas the other representing the honeycomb network.

4.2. Pore size measurement

The pore size of freeze-dried SF/HA scaffolds were measured by ImageJ software as described above. The results of the pore size measurement are shown in figure 14. The result demonstrated that SF80 scaffolds show the significant highest pore size among the group.

4.3. Mechanical properties

Because biomaterials for tissue engineering were used in wet condition, the mechanical property of chemically cross-linked SF/HA scaffolds were tested after submerged in PBS, as shown in table 5. Pure fibroin scaffolds and blends scaffolds showed the sponge-like behavior. Load and stress at the limitation of 40% strain were significantly reduced when increase hyaluronic acid content. According to these results, the stiffness and compressive modulus were also reduced with the increase of hyaluronic acid content. Decreasing in compressive modulus may occur from the increment of pore size and molecular properties of

hyaluronic acid. Interestingly, the shape of all scaffolds reverted completely after load withdrawal.

4.4. Swelling property

The water-binding capacity of scaffolds is an essential indicator for biomaterial properties evaluation. The water uptake of 3Ds SF/HA scaffolds significantly increased when increase the content of hyaluronic acid (table 6). The result suggesting that the hydrophilicity of scaffolds was improved when adding hyaluronic acid.

4.5. Immunophynotypes and multipotency of HUMSCs

HUMSCs were successfully isolated from umbilical cord tissue (Wharton's jelly). The extruded cells showed a distinct fibroblastic-like morphology and MSC-like phenotype (data not shown). Flow cytometry revealed that the cells isolated from umbilical cord tissue were positive for mesenchymal markers CD10 and CD 13 and negative for hematopoietic marker CD34 and CD45 (data not shown).

The differentiation capability of the cells isolated from umbilical cord tissue was also investigated. For the osteogenic differentiation, the HUMSCs were treated with osteogenic condition medium for 21 day and then observed by the deposition of calcium phosphates which specifically be stained with alizarin red. In this study, a large number of orange-red matrices by alizarin staining were considerably observed on day 21 after initiation induction, indicating the formation of mineralized extracellular matrix which secreted from the induced HUMSCs (figure 15A). The adipogenesis of HUMSCs was also induced by culturing the cells in adipogenic differentiation media for 14 days. Our experiments showed that positive oil red O staining was markedly observed in cytoplasm and extracellular compartment of the induced HUMSCs, implicating the accumulation of lipid vacuole in adipogenic inducing cells (figure. 15B). After 14 day of chondrogenic induction of HUMSCs, the positive staining of alcian

blue was also observed which indicated that the expanded HUMSCs could differentiate to the chondrogenic lineage (figure 15C). Thus, the explanted HUMSCs were appropriated to the thereafter examination for the cartilage regeneration.

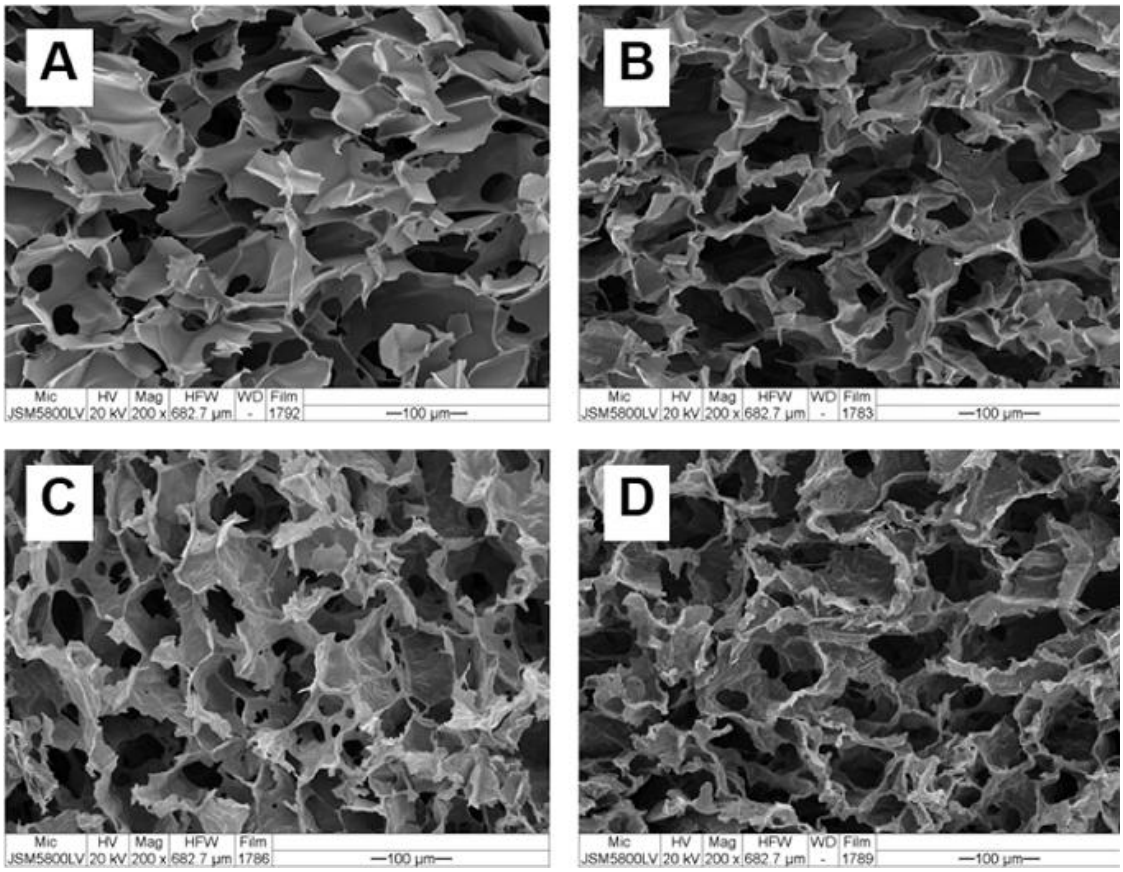


Figure 13. Scaffold morphology observed by SEM after treatment with methanol: A. SF100; B. SF90; C. SF80; and D. SF70.

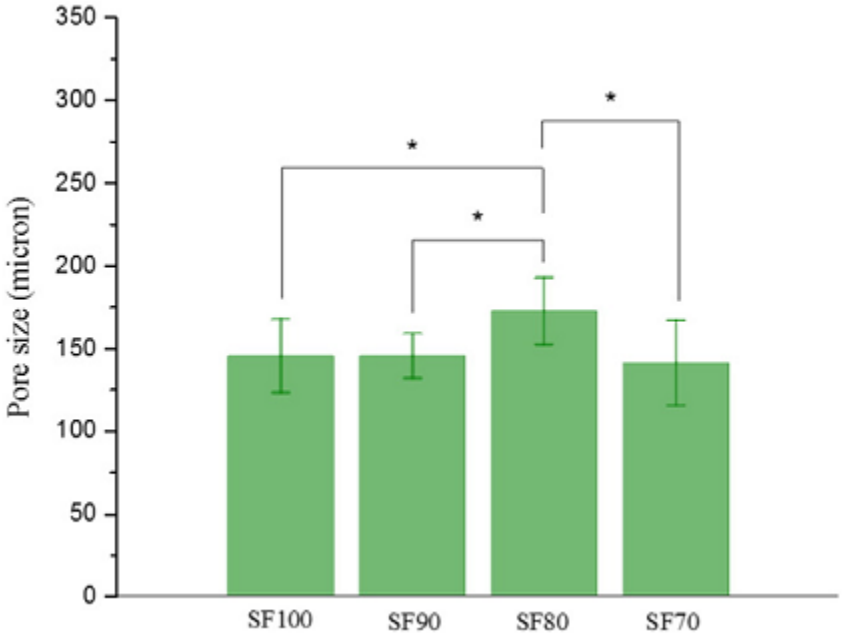


Figure 14. Pore sizes of the SF scaffolds. *P < 0.05 was accepted as statistically significant.

Table 5. Mechanical properties of chemical cross-linked SF/HA scaffolds after swelling in PBS

Sample	Load at limit (N)	Stress at limit (kPa)	Stiffness (N/mm)	Compressive Modulus (kPa)
SF100	0.72±0.02	5.01±0.16	0.53±0.06	33.55±3.85
SF90	0.41±0.03	2.65±0.21	0.17±0.01	9.04±0.78
SF80	0.24±0.01	1.38±0.08	0.09±0.01	4.88±0.17
SF70	0.13±0.02	0.59±0.01	0.05±0.01	2.21±0.11

Value are average ± standard derivation (n = 6)

Table 6. Swelling ratio and water uptake (%) of SF/HA scaffold in PBS

Sample	Swelling ratio	Water uptake (%)
SF100	20.63 ± 1.85	95.35 ± 0.43
SF90	25.53 ± 1.03	96.22 ± 0.14
SF80	30.17 ± 1.27	96.79 ± 0.13
SF70	40.07 ± 1.62	97.56 ± 0.09

Value are average ± standard derivation (n = 6)

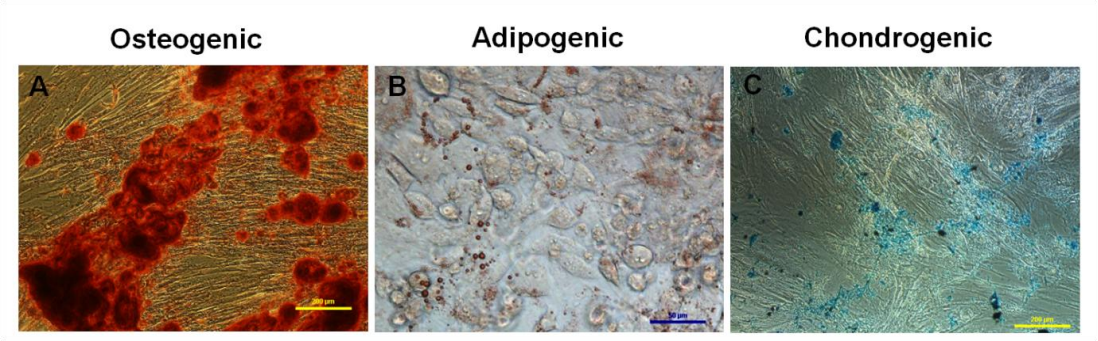


Figure 15. The multipotency of HUMSCs in this study

4.6. HUMSCs proliferation, morphology and cell viability in SF/HA scaffolds

To evaluate HUMSCs proliferation on silk/HA scaffolds. Resazurin reduction assay was achieved (figure 16). Principally, the resazurin dye in culture medium was reduced by mitochondrial enzymes of living cells to its metabolized resorufin (137). This assay measures the fluorescence intensity of the red-yellow light emitted from resorufin when it excited by green light. Thus, the fluorescence intensity value directly relates to cellular metabolism and number of cells. It was observed that in the cases of SF100 and SF90 silk/HA scaffolds, the fluorescence intensity of resorufin were comparable at day 7, 14 and 21. However, the proliferation values of HUMSCs in SF 90 were less than SF100. When increase in the percentage of HA in scaffolds, lower cell proliferation was observed, in case of SF 80 and SF70 silk/HA scaffolds. The lowest cell proliferation was estimated in the SF70 scaffolds which showed the highest of water content. This result suggested that the hydrogel-like behavior of scaffolds had an effect to the metabolism of HUMSCs.

The attachment and morphology of HUMSCs under chondrogenic condition within scaffolds was observed by Phallotoxin staining (figure 17). In the 4^{th} week, HUMSCs in SF80 and SF70 scaffolds was condensed to spheroid shape. In contrary, the cells in SF100 and SF 90 showed the elongated, flatten morphology and eventually spreading throughout scaffolds.

HUMSCS viability during chondrogenesis in silk/HA scaffolds was detected by double staining of FDA and PI at day 28 after chondrogenic induction (figure 18). Fluorescence micrographs showed that HUMSCS embedded in SF100 and SF90 under chondrogenic condition displayed a distinct fibroblastic-liked morphology. In contrast, the cells in SF80 and SF90 aggregated as a microsphere by various sizes. Live and dead staining revealed that almost HUMSCs in all scaffold types were viable throughout the culture period. The viable of cells were indicated by the present of green fluorescence light which resulting from the conversion of non-fluorescence FDA into the green fluorescent metabolite by the

esterase enzymes of living cells. Surprisingly, the dead signal of red nuclei staining by PI was not observed. This result indicating that the all fibroin-based scaffolds are non-toxic and suitable for cell cultivation for chondrogenic support.

4.7. Histological and immunohistological analyses of chondrogenesis in SF/HA scaffolds

Histological staining by H&E and alcian blue was performed in both pellet and scaffolds culture experiments. Histological sections revealed the highly distribution of a typical spindle-shaped characteristic of MSCs in SF100 and SF90 scaffolds (figure 19), however, some characteristic of cells embedded in each scaffolds are quite different. The cells in SF100 are homogenously distributed, but in SF90 the cells condensed in some areas. For the extracellular matrix production, HUMSCs embedded in SF 90 considerably produced more extracellular matrix than those in SF100 which were evaluated by the higher positive staining of eosin and alcian blue. When increase HA content, the spherical morphology cells was observed in both SF80 and SF70. The condensed spheroid showed the accumulation of extracellular matrix inside which determined by H&E and alcian blue staining. These characteristic were resembled to pellet culture of HUMSCs. The present of chondrogenic-specific proteins including collagen type II and aggrecan in all cultures were determined by immunohistochemistry at day 28 after induction (figure 20-21). The results showed that both of collagen type II and aggrecan were positively stained in all experiments suggesting the HUMSCs underwent chondrogenic differentiation. Consisting to the staining of alcian blue, the immunostaining by antibodies against collagen type II and aggrecan in SF90 were more intense than SF100. Likewise, the spheroids in both SF80 and SF70 also expressed both collagen type II and aggrecan which resembled to the result obtained from pellet culture. The results obtained by immunohistochemistry were significantly consistent with that obtained by histochemical analysis of alcian blue staining.

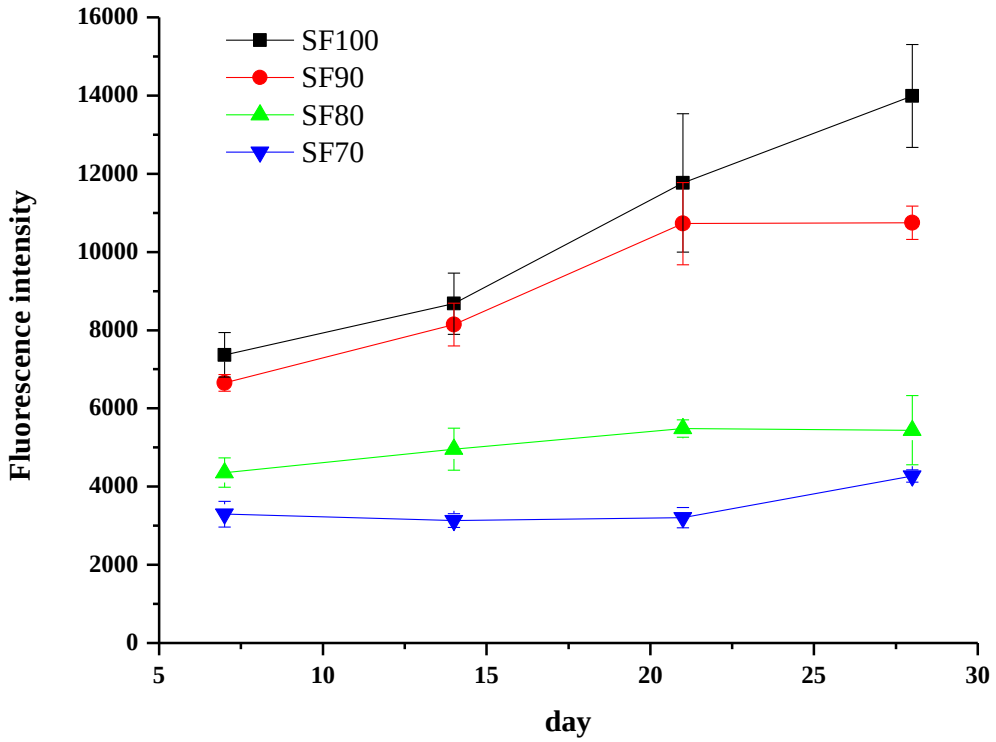


Figure 16. HUMSCS proliferation in the different types of silk/HA scaffolds (SF100 ■, SF90 ●, SF80▲ and SF70 ▼ n = 6) on day 7, 14, 21 and 28 by resazurin reduction assay. Initial cell seeding density was $2.5x10^5$ cells/scaffolds. Data represented as average ± standard deviation ($p < 0.05$).

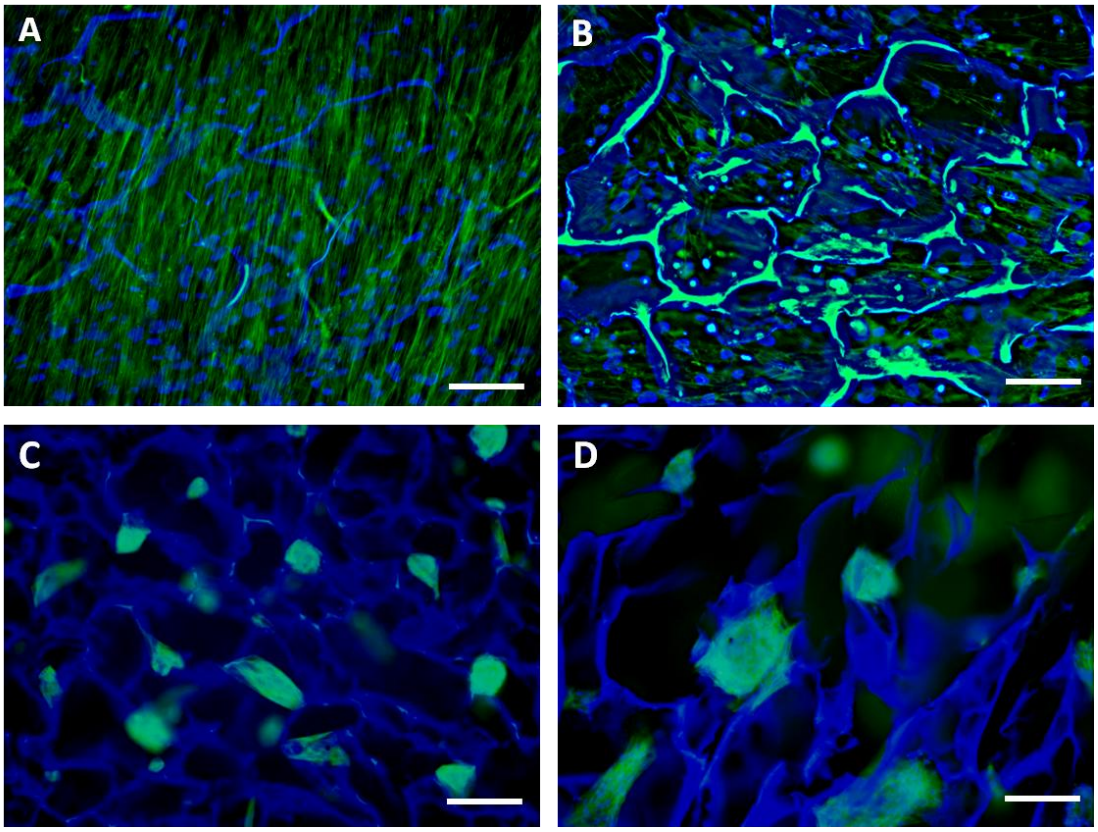


Figure 17**.** Fluorescence micrographs of HUMSCs cultured on silk/HA scaffolds (SF100 [A], SF90 [B], SF80 [C] and SF70 [D]) stained with FITC FITC-phalloidin for actin filaments (green) and H33342 for nuclei (blue). Scale bar represent 100 µm.

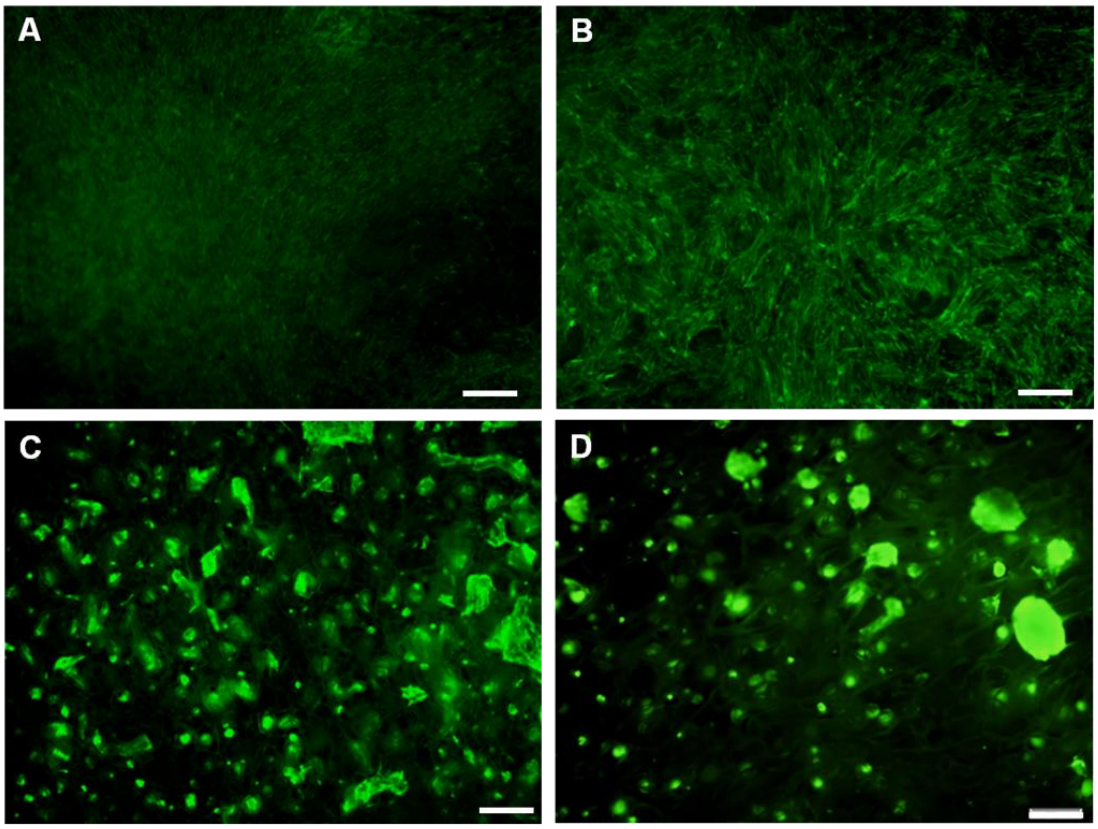


Figure 18. Cell viability during chondrogenesis. At day 28 after chondrogenic induction, the viability of HUMSCs in constructed scaffolds (SF100 [A], SF90 [B], SF80 [C] and SF70 [D]) was evaluated by double-staining with FDA and PI. Green staining indicates viable cells, scale bar represent 200 μm.

4.8. Real-time PCR for the analysis of chondrogenesis in SF/HA scaffolds

The expression of chondrogenic-specific genes including col2a, agg and sox9 were analyzed by time-course real-time PCR (figure 22). Scaffolds-embedded cells cultured under chondrogenic condition for 1, 2 and 3 weeks were investigated. Data indicated that all of chondrogenic marker was significantly up-regulated in all scaffold types. The level of gene expression for collagen type II, aggrecan and Sry-type high mobility group box transcription factor 9 were increased with the increase of hyaluronic acid content. The highest of col2a, agg and sox9 was detected on day 21 after chondrogenic induction of SF80, however, under statistical analysis there are not significantly different between SF80 and SF70. The results obtained by real-time PCR were consistence to the results obtained from immunohistochemical analysis.

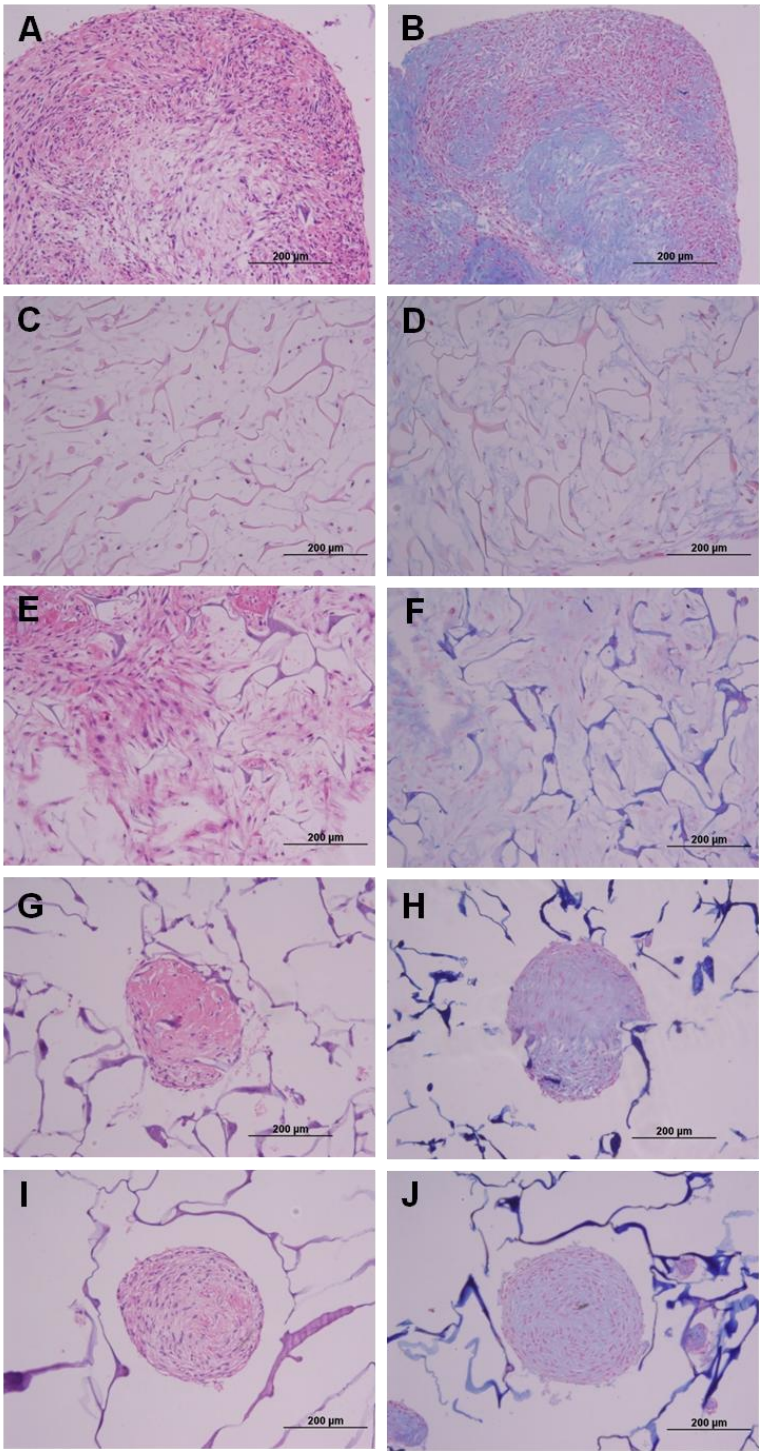


Figure 19. H&E and alcian blue staining. Chondrogenic differentiation of pellet cultured of HUMSCs (A) and HUMSCs embedded in silk/HA scaffolds was evaluated by H&E staining at 4th week after chondrogenic induction (SF100 [C], SF90 [E], SF80 [G] and SF70 [I]) and alcian blue staining at 4th week after initial induction (pellet cultured [B], SF100 [D], SF90 [F], SF80 [H] and SF70 [J]). Scale bars represent 200 µm.

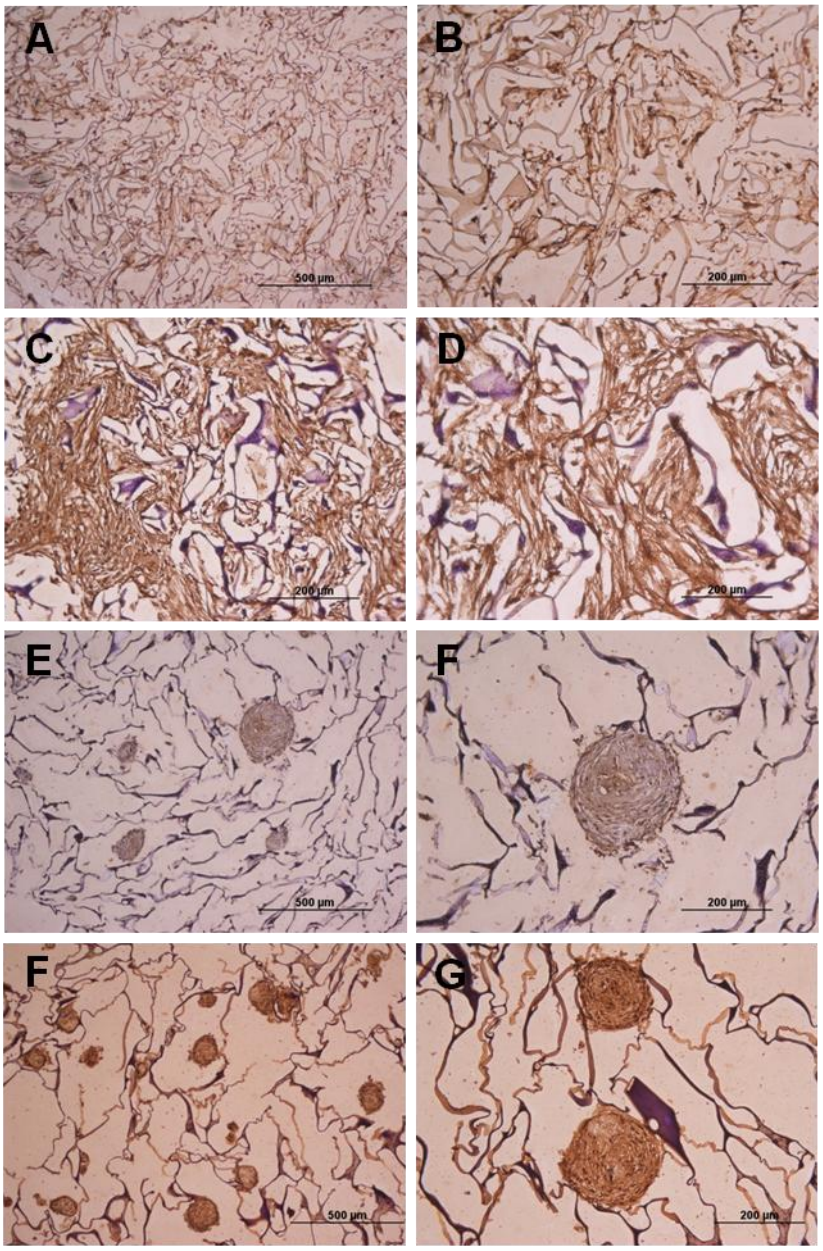


Figure 20. Immunohistological staining for the expression of collagen type II of induced HUMSCs. Chondrogenic differentiation of HUMSCs embedded in silk/HA scaffolds was evaluated by Immunohistochemistry at 4th week after chondrogenic induction (SF100 [A, B], SF90 [C, D], SF80 [E, F] and SF70 [I, J]). Scale bars represent 500 µm and 200 µm respectively

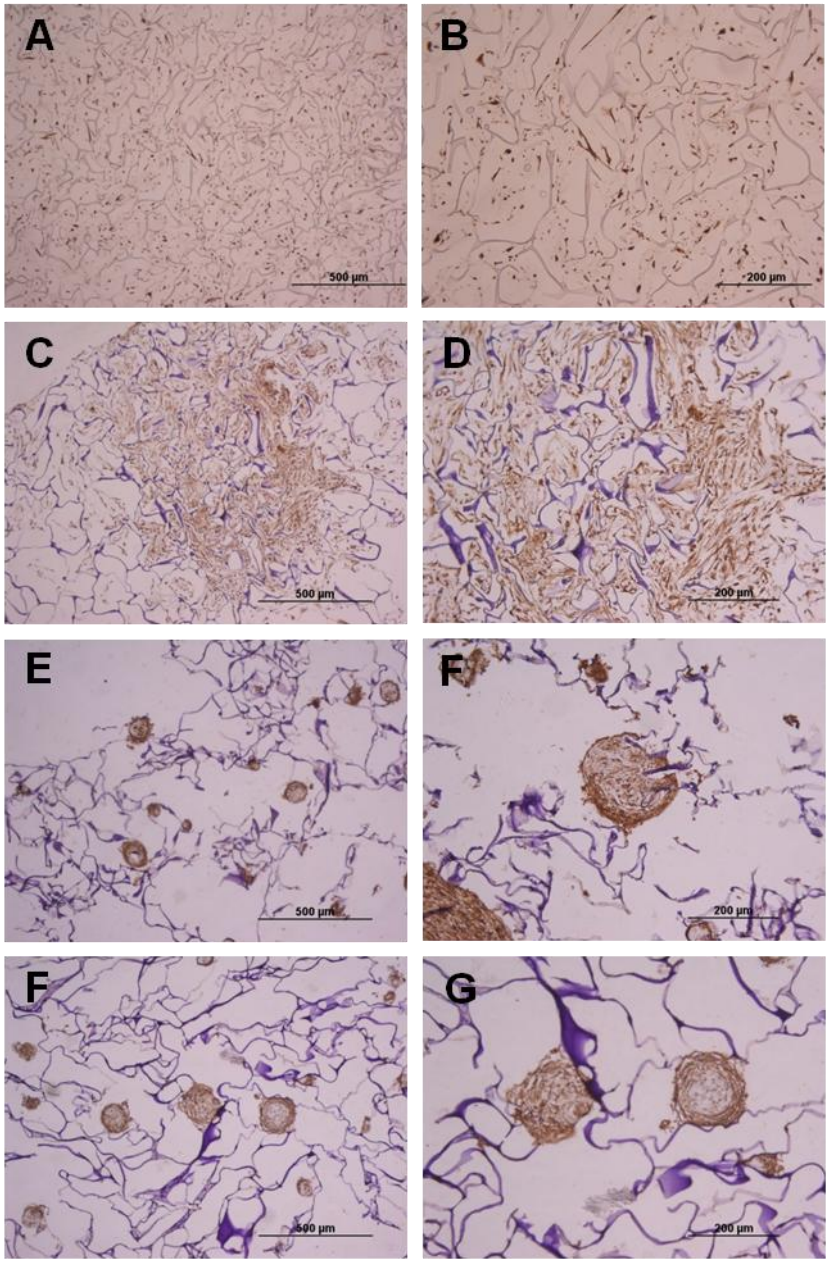


Figure 21. Immunohistological staining for the expression of aggrecan of induced HUMSCs. Chondrogenic differentiation of HUMSCs embedded in silk/HA scaffolds was evaluated by Immunohistochemistry at 4th week after chondrogenic induction (SF100 [A, B], SF90 [C, D], SF80 [E, F] and SF70 [I, J]). Scale bars represent 500 µm and 200 µm respectively.

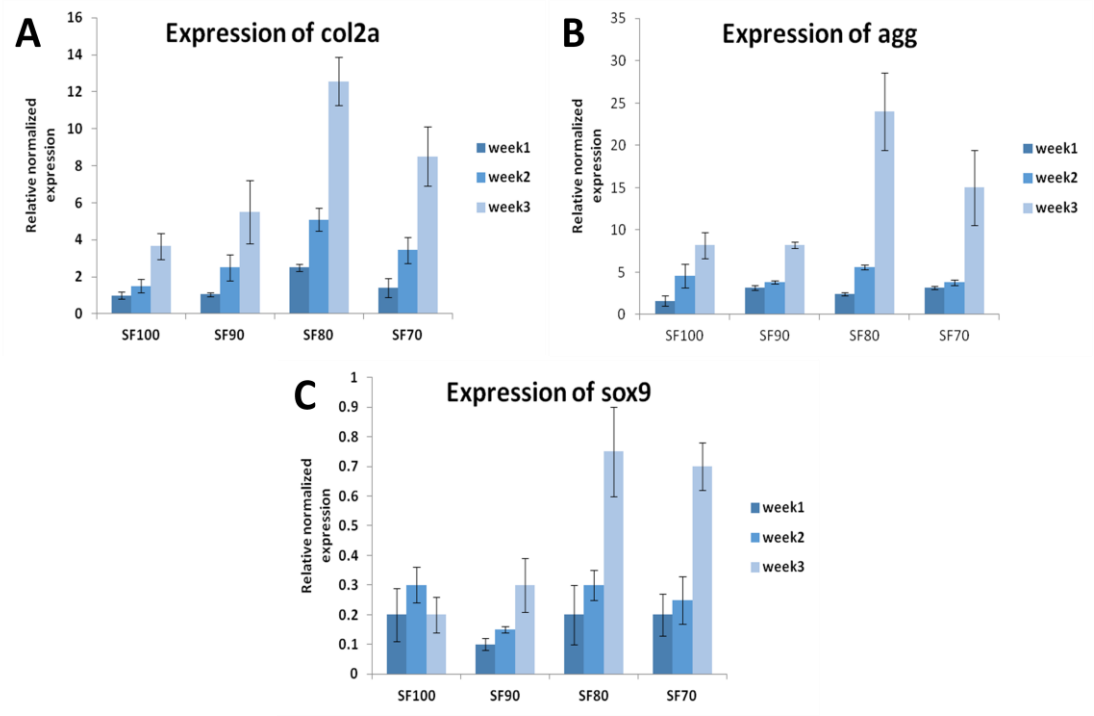


Figure 22. Relative normalized expression of chondrogenic-specific gene to GAPDH of HUMSCs embedded in SF/HA scaffolds at day 7, 14 and 21after chondrogenic induction, (A) col2a, (B) agg and (C) sox9. Value are average ± standard derivation (n = 6)

CHAPTER 5

DISCUSSION

The self-remodeling of cartilage is relatively scarce as resulting of the deficiency of blood and nerve supplied which are the key components in wound healing process. Thus, the reconstruction and regeneration of cartilage defects or injuries is the most recently interested clinical problems due to the high incidence of osteoarthritis in elderly and joints damage in athletes. The present articular cartilage repairing techniques (73), including microfracture, autologous chondrocyte implantation and scaffold-based approaches have been investigated and applied in clinic. Unfortunately, these therapeutic approaches for chondral defect under surgical intervention are not able to complete restoration and long-term maintenance (129). However, the limitation of the available standard techniques for treating cartilage lesions could be overcome by tissue engineering strategies either cell-based (with and without scaffolds) or whole-tissue transplantation.

This study, we investigated whether on cell-based (HUMSCs) with scaffolds (SF/HA) for cartilage regeneration. In general, for the designation of cartilage scaffold, the porous of mimic extracellular matrix acts as a polymeric cage for chondrocyte and also provide mechanical and physical clues for stem cell regulation and differentiation into cartilage cell. Thus the morphological mechanical and physical properties of native tissue are the major consideration when creating the mimic cartilage scaffold. Our biocompatible fabricated scaffolds were high in mechanical flexibility and providing an effective microenvironment for stem cells to proliferate and differentiated inside. In the view point of morphological property, the thickness of pore were markedly increased regarding to the content of HA in scaffolds with the corresponding the roughness of pore wall which increased when increase HA. The non-homogeneous structure was often occurring in the blended polymers (59). Thus the pore formation and pore architecture of silk fibroin-based scaffolds could be controlled by

proportional changes of hyaluronic acid in the scaffolds (133). Interestingly, the roughness of wall surface can enhance tissue regeneration by inducing the attachment and proliferation of cells as previous mention (138, 139). Moreover, the blend scaffolds represent the regularly continueous pore wall, whereas the pure fibroin scaffold show irregular and broken wall in some parts.

The pore size of scaffolds was also measured; the mean pore size of SF80 was significantly largest. This observation is probably the resulting of the disturbance of SF molecules into regular structure of SF80 by HA. The increment of pore size could enhance the elasticity of scaffold (139), especially in SF80. In the group of low content of HA (SF90) and pure SF (SF100), the pore size did not different indicated that there are no any interfere in SF molecule by HA. However, pore size in SF70 was smaller than SF80 without statistically different when compare to SF100 and SF90. Since HA can induce molecular interaction in SF molecule (140), thus result of pore size in SF70 which have the highest content of HA could be resulting from the extension retardation of SF molecule by HA. Interestingly, the result of pore size in all group were in the range for the culturing of chondrocyte (141) (90mm-300mm).

Other important clues of tissue regeneration are mechanical properties (142, 143). The content of hyaluronic acid in silk fibroin-based scaffold also has a great influencing on the mechanical property of scaffolds. As shown in table , the reduction of load, stress and also compressive modulus of the sponge-like scaffolds, when increase the content of HA in blend, may occur from the superior in molecular hydrophilicity of HA that could induce softness of the scaffolds (144). All scaffolds can maintain their shape after load withdrawal in wet condition. The elastic and soften behavior of SF/HA scaffolds were resembled to the mechanical characteristic of cartilage tissue (145). Therefore, SF/HA scaffolds were suitable for cartilage tissue engineering.

In addition, the superior in hydrophilicity of HA also improved the water uptake of the scaffolds. The swelling of silk based scaffolds in aqueous solution were increased when increase the content in mixtures (Table 3). Moreover, The SF80 and SF70 scaffolds were highlighted when incubated in aqueous condition either in PBS or cell culture medium the bulk morphology of them was favorable like hydrogel. Naturally, the extracellular matrix of cartilage represents high water uptake and swelling. Thus, the water-binding capability of scaffolds is essential indicator for cartilage tissue engineering.

Bone marrow-derived MSCs have been most recently use for cartilage regeneration in clinic (73). However, the isolation of bone marrow stem cells is an invasive procedure which made some discomfort to donor. This study, we isolated the previously identified mesenchymal stem cells derived from umbilical cord (146). Our isolated HUMSCs exhibited a distinct fibroblastic-like morphology in culture vessel and expressed the identical MSC markers on their surface. In addition, these cells can differentiate along osteogenic, adipogenic and also chondrogenic lineages.

Stem cells from birth associated tissue, especially from umbilical cord has a great potential for treating the musculoskeletal disorders. MSCs derived from umbilical matrix is a promising source for clinical use to treat various musculoskeletal disorders due to the differentiation potential along chondrogenic, osteogenic, adipogenic, myogenic and neurogenic lineages (119). HUMSCs can be easily harvested from tissue that should be discarded as medical waste without ethical problems. Especially stem cells derived from umbilical cord matrix had been recently investigated for cartilage regeneration. For the cartilage regeneration, the initial study of the pellet culture of wharton's jelly derived stem cells under chondrogenic condition which supplemented with TGF-b1 representing the deposition of collagen type II in pellets (147). Recently, Arufe *et al*. (148) evaluated the expression of chondrogenic markers through the spheroids of human umbilical cord-derived

stem cells. They found that the expression of collagen type II, and collagen type X were early detected at day 4 after chondrogenic induction and their expression were increased by the time. In addition, for the *in vitro* chondrogenesis of HUMSCs incorporated with scaffolds. Chen *et al* recently seeded HUMSCs in collagen type I hydrogel and evaluated the chondrogenic expression (149). They found that HUMSCs underwent chondrogenesis in hydrogel with the highly expression of collagen type II, aggrecan, COMP (cartilage oligomeric matrix protein) and also sox9.

This work also evaluated the possibility of HUMSCs incorporated with SF/HA scaffolds in the comparison with pure-fibroin scaffolds for cartilage tissue engineering. Although, HUMSCs embedded in all of our fabricated scaffolds were viable over the culture period. However, the observation of cells proliferation in each culture by resazurin assay was different. The metabolic activity which was interpreted from the present of resorufin in SF100 and SF90 cultures increased by the time (Fig 4), but the metabolic activity of SF80 and SF70 culture were stable over time. As noted, the oxidative phosphorelation of differentiated MSCs pellets under chondrogenic condition were lesser extent than standard culture and under osteogenesis (150). Interestingly, the spheroids of HUMSCs were observed throughout of SF80 and SF70 scaffolds (Fig 5 and 6) and the morphology and extracellular matrix accumulation also resembled to pellet culture of HUMSCs under chondrogenic condition (Fig 7).

The pre-cartilage condensation is the intermediate state of MSCs that underwent chondrogenesis (151). After this stage, the spheroids likely to express their chondrogenic extracellular matrix including, collagen type II, IX and aggrecan and became hypertrophy. The primarily chondrocyte that observed in SF80 and SF70 scaffolds revealed the higher expression level of collagen type II aggrecan and sox9 that those in SF100 and SF90. Additionally, the high intensity of collagen type II and aggrecan in the SF/HA scaffold after

28 day culture observed by IHC was found to be consistent with the up-regulation of the chondrogenic gene markers, collagen type II and aggrecan, whereas the expression levels of these markers were low in the pure silk fibroin scaffold suggesting that hyaluronic acid is likely to be a key component for the promotion of chondrogenesis in the silk fibroin scaffold. The presence of hyaluronic acid in the scaffolds might regulate the chondrogenesis of HUMSCs via the interaction through their specific receptor CD44 which provided an appropriate signals that preserving the chondral phenotype during chondrogenesis (152). Although, hyaluronic acid might enhance chondrogenesis when compared to pure scaffolds, but the pre-cartilage formation were not observed in SF90. From these results we can conclude that the content of hyaluronic acid which reflected to the hydrophilicity of scaffolds might also facilitate the pre-cartilage formation within scaffolds. Thus, the obtained result obviously indicated that presentation of different form of differentiated HUMSCs may influenced by porous structure and HA content in scaffolds and also the differences in mechanical and swelling property.

Visco-elastic SF/HA scaffold may provide an appropriate microenvironment for the chondrogenesis of HUMSCs. However, the component of scaffolds could be adjusted or the scaffolds should be decorated with other active molecules that can promote the neocartilage formation from HUMSCs. Moreover the HUMSCs embedded in SF/HA scaffolds should be investigated further in the *in vivo* study.

CHAPTER 6

CONCLUSIONS

The cellular scaffolds based on SF/HA in combination with human umbilical cord mesenchymal stem cells were created as a candidate for treatment of osteoarthritis. Scaffolds morphological, mechanical and physical properties were investigated. The result revealed that the biomimetic scaffolds influenced the chondrogenesis of such stem cells. Especially, the SF80 and SF70 demonstrated the predominated results of chondrogenic support. The pre-cartilage condensations of stem cells were observed in scaffolds. Notably, the SF/HA scaffolds show the appropriate biological performance for cartilage tissue engineering and hold promise in osteoarthritis treatment using surgical technique. However, the *in vivo* performances need to be addressed for proving the suitability for cartilage tissue engineering.

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