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Development of platelet-rich fibrin (PRF) as biodegradation scaffold for application in cartilage engineering

富含血小板纖維蛋白在軟骨組織工程中作為生物降解性細胞支架的製備與應用

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與時遷移，看似漫長的兩年研究生涯轉眼間即將劃上句號。對於大學時期從沒任何實驗室經驗的我，雖然面對的是個全然陌生的環境，但自己卻是幸運的，有盡心盡力的師長與認真熱心的同學一路扶持。

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中文摘要

軟骨組織內缺少血管與神經，自我修復能力有限，因此對於軟骨損壞的治療上是一大挑戰。骨關節炎 (Osteoarthritis，OA)，是一種局部性關節軟骨退化病變，主因為關節軟骨組織新生合成速度趕不上分解破壞速度所導致，普遍好發於過度負重及老化。本篇論文研究目的在利用軟骨組織工程原理，以 Platelet-rich fibrin (PRF) 合併 fibrin 方式開發新型細胞生長支架 (scaffold) ，於體外培養出健康軟骨細胞，再經由注射方式修復受損之關節軟骨，探討此 fibrin＋PRF 複合生長支架對於軟骨細胞的增生以及分化能力有否有所助益。富含血小板纖維蛋白 (PRF) 是近年來新發現由血液中萃取的血小板濃縮物，本身富含生長因子和細胞刺激素，尤其是 PDGF-BB、IGF-1、TGF-β1、BMP-2，這些蛋白對於軟骨細胞的增生與分化有所助益。實驗中製作了兩種 fibrin scaffold ，一種是以纖維蛋白原 (fibrinogen) 和 凝血酶 (thrombin) 合成 fibrin gel，另外以 PRF exudates 作為 fibrin gel scaffold 的添加劑，生成 fibrin + PRF 複合生長支架。此外 fibrin scaffold 作成兩種型態，膠體形式與以冷凍乾燥方式做成海綿狀物質。實驗裡應用人類軟骨瘤細胞株 (human chondrosacrcoma cell, SW-1353) 和初代人類軟骨細胞 (primary human chondrocyte) 培養模式，將細胞分別培養在 fibrin gel、添加 PRF 溶液之 fibrin 複合膠體以及 agarose gel 的二維 (2D) 與三維 (3D) 三種不同生長支架中，評估軟骨細胞的增生與分化情形。實驗中發現從血液中分離所得 PRF 降解後分泌液與軟骨細胞生長相關生長因子濃度平均高於其他血液裡萃取之衍生物 (serum, plasma, fibrin)。我們由觀察外觀和使用電子顯微鏡 (SEM) 比較兩種型態 fibrin sponge scaffold 內部結構。結果顯示 gel 狀 fibrin scaffold 較 sponge 狀結構來的堅固且細胞生長效果較佳。然而軟骨細胞在 agarose gel scaffold 中因不貼附所以生長效果最差。細胞培養在三種不同 scaffold 中經過4、8、12天三個時間點作觀察，發現軟骨細胞在添加 PRF exudates 後的 fibrin scaffold 上 type II collagen 與 aggrecan 的 mRNA 表現量較純粹

fibrin scaffold 有明顯上升的趨勢，證實軟骨細胞利用此複合 3D 生長支架 re-differentiation 的效果更好。組織切片方面，經由 Alcian blue 與 PAS 染色結果得知，培養於 3D fibrin + PRF scaffold 中軟骨細胞產生之蛋白多醣 (proteoglycan) 與糖胺聚醣 (glycosaminoglycan，GAG) 量優於在 fibrin scaffold 中。綜合結果顯示出 PRF 因本身富含生長因子與細胞刺激素，使添加 PRF 後的 fibrin gel，更能促進軟骨細胞的增生及分化。未來在組織工程中，將 PRF 運用於軟骨修復上，搭配 fibrin gel 作為有效的立體生長支架使軟骨細胞能有效增生且維持細胞於分化狀態，進而達到軟骨再生效果，用於治療 OA 是具有潛力的。

關鍵字：骨關節炎 (osteoarthritis，OA)、軟骨細胞 (chondrocyte)、細胞支架 (scaffold) 、纖維蛋白 (fibrin)、富含血小板纖維蛋白 (platelet-rich fibrin，PRF)、瓊膠 (agarose)、糖胺聚醣 (glycosaminoglycan，GAG) 、聚蛋白多醣 (aggrecan)、膠原蛋白 (collagen)

Abstract

Because of articular cartilage has a limited ability for self- repair. Thus, treatment of cartilage lesions is a challenge. Osteoarthritis (OA), is known as degenerative joint disease and a type of arthritis that is caused by the breakdown and eventual loss of the cartilage of one or more joints. This thesis investigates to incorporate PRF that involves developmental signals into fibrin for use in cell biological studies and as a regeneration matrix employing tissue-engineering (TE). The goal is to explore the fibrin gel that adds PRF scaffold for in vitro culture and in vivo of chondrocytes proliferation and differentiation. Platelet-rich fibrin (PRF) is a new generation of platelet concentration, it's abundant in platelet cytokines, like platelet-derived growth factors (PDGF-BB), insulin-like growth factor-1 (IGF-1), transforming growth factor (TGF-β1) and bone morphogenetic proteins (BMP-2). They have the ability of chondrocytes proliferation and differentiation. In the thesis, fibrin scaffolds have two types. One is produced from bovine fibrinogen and thrombin that mix to fabricate the fibrin gel, and the other is add PRF from human blood by centrifugation without anticoagulant into fibrin scaffold. And the fibrins are made two forms, the fibrin gel and it is to make a fibrin sponge by freeze-drying. We set up the primary human chondrocytes and SW-1353 . The primary chondrocytes and SW-1353 are embedded and developed in these scaffolds. Additionally, the agarose scaffold is to be the control. Three types of three-dimension (3D) and two-dimension (2D) culture are fibrin, fibrin with PRF and agarose scaffold are evaluated，which is the adaptive one to enable the chondrocytes proliferation and differentiation . We compare SW-1353 and primary human chondrocytes on 2D and in 3D of three kinds of scaffold. In order to carry out a comparative study, we undertake the inner microstructure of fibrin, PRF sponge that has an interconnected pore structure is observed by scanning electron microscope (SEM). And we observe cell morphology by using microscopy. To

quantify the concentration of growth factors in the PRF exduates, PDGF-BB, IGF-1, TGF-β1, and BMP-2 are stimulators that correlated with proliferation and differentiation by ELISA. MTT assay and RT-PCR are used for estimate cell survivability and the mRNA expression of type II collagen and aggrecan. To detect the glycosaminoglycan (GAG) from chondrocytes are in different scaffold by PAS and Alician blue staining. The results showed that the chondrocytes on 2D and in 3D fibrin + PRF scaffold structure could provided the more available proliferation and differentiation than cells just on 2D and in 3D fibrin or agarose. It is concluded that the fibrin with PRF gel including abundant cytokines and growth factors is a promising three-dimension scaffold of cells for cartilage tissue engineering.

keywords : Osteoarthritis (OA)、chondrocyte、scaffold、 fibrin、 platelet-rich fibrin (PRF)、agarose、glycosaminoglycan 、aggrecan、collagen.

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Introduction

Chondrocytes produce and maintain the cartilaginous matrix, which consist mainly of collagen and proteoglycans which perform matrix-generation and maintenance functions. Osteoarthritis (OA) is a chronic degenerative joint disease because of imbalance between catabolic and anabolic chondrocyte activity. It results in degradation of cartilage and inflammation in joint. Factors that may cause osteoarthritis include being overweight, getting older and injuring a joint. Primary osteoarthritis is mostly related to aging. With aging, the water content of the cartilage increases and the protein make up of cartilage degeneration. Because of cartilage has no vascular, therefore, possesses limited for repair and regeneration in itself. The high prevalence of OA and the poor intrinsic healing capacity of articular cartilage engender a demand for cell-based strategies for cartilage repair **[1]**. In several applications, including surgical implants, gene transfer with growth factors by virus and something else. Tissue engineering has already made a successful transition from a scientific method to a clinical procedure **[2]**. Cartilage tissue engineering was proposed as a cartilage replacement method, because it is able to overcome many of the problems associated with traditional cartilage replacement methods. It is the construction, repair or replacement of damaged or missing tissue in humans and other animals. This engineering may take place within the animal body or as tissue constructs to be made in a bioreactor for later grafting into the animal **[3]**.

Tissue engineering procedures require the transplantation of functionally active cells within supportive carrier matrices **[4]**. It is involved the three key ingredients: harvested cells, recombinant signaling molecules, and three-dimensional (3D) matrices to improve or replace biological functions. Cells are often implanted or seeded into an artificial structure (scaffold

) capable of supporting three-dimensional tissue formation. There are numerous factors to accomplish successful tissue engineering. The most important aspect of cartilage tissue engineering is the design of a scaffold structure that controls three-dimensional shape and guides tissue development **[5].**

Successful tissue engineering is dependent on numerous factors, but adequate scaffolds are among the most important prerequisites for stable three–dimension and histiotypic tissue **[2]**. A well-designed scaffold for tissue regeneration is one of the fundamental tools to provide an environment necessary for cell differentiation and affect the final shape of tissue regenerated. in vitro and in vivo. The cartilage tissue engineering to treat OA is for the cells to attach to the scaffold, multiply, differentiate, and organize into normal, healthy bone as the scaffold degrades. Suspending chondrocytes in a three-dimensional matrix, similar to their natural environment, can permit the cells to retain their native phenotype and produce their extracellular components. Hydrogel scaffolds appear to satisfy this requirement **[6].**

Fibrin possess several important features for the scaffold material for cell proliferation and differentiation, it is biocompatible, biodegradable and has an inherent affinity for various biological surfaces. These features are essential for the scaffold matrix used in tissue engineering. In recent years, fibrin gel is utilized for different applications in the wide field of tissue engineering. It is a biopolymer matrix commonly used for surgical hemostasis and tissue sealing and is known to be critical in the normal wound healing process **[7-9].** It also plays an important role in regeneration in the developed organism. It is derived from plasma coagulation proteins. Commercially available fibrinogen and thrombin are combined to form a fibrin gel. The fibrin hydrogel, also known as fibrin tissue adhesives. They are the most successful of the tissue sealants in terms of tissue compatibility, biodegradation

and clinical utility **[10]**. The natural polymer is used for tissue engineering increasingly. Hydrogels, appears to also satisfy the need of a perfect filling of the lesion, as they can be easily applied onto an irregular defect **[6].**

The physiological composition of fibrin makes it a particularly well-suited biomatrix for tissue engineering applications. Fibrin has been long used as an effective scaffolding material to grow a variety of cells and tissue constructs. It has been utilized mainly as a hydrogel in varying concentrations to provide an environment in which suspended cells work to rearrange the fibers and lay down their own extracellular matrix **[11]**. An important fibrin characteristic is an increasing instability and solubility over time in vitro and in vivo, due to fibrinolysis. Rapid degradation could be an advantage in wound sealing or other surgical applications as well as for cell and growth factor delivery. However, this can represent a problem for use as a shape-specific scaffold in tissue engineering **[12]**. So it is necessary to make up a cell scaffold of fibrin which is firm and long term stable, aiming at the tissue engineering.

The new biomaterial, called platelet-rich fibrin (PRF), is a second-generation platelet concentration and an autologous cicatricial matrix. It belongs to a new generation of platelet concentrates geared to simplified preparation without biochemical blood handling. **[13]** Because of platelets contain abundant growth factors, like PDGF, TGF-β1, IGF-1, and BMP-2. PRF would be able to progressively release these cytokines during fibrin matrix remodeling. It was first developed in France by Choukroun et al. for specific use in oral and maxillofacial surgery. Clinical data reveal that this biomaterial would be a favorable matrix for the development of a coherent healing without inflammatory excess **[14, 15]**. This technique requires neither anticoagulant nor bovine thrombin (nor any other gelling agent). The PRF

protocol makes it possible to collect a fibrin clot charged with serum and platelets. **[13]** Because of its weak thrombin concentrations imply a very significant percentage of equilateral junctions. These connected junctions allow the establishment of a fine and flexible fibrin network able to support cytokines enmeshment and cellular migration. PRF platelet cytokines remain trapped in the fibrin meshes, and probably even in the fibrin polymers **[16]**.

Moreover, this three-dimensional organization will give great elasticity to the fibrin matrix: It is in a flexible, elastic, and very strong PRF membrane. These fibrin biotechnologies therefore use different polymerization modes which imply very different biologic integration mechanisms. The platelet-derived growth factor (PDGF) is a kind of growth factors in the fibrin, It acts on many cells, especially mesenchymal cells, as a mitogen and a chemotactic factor **[17]** .And it has been shown that positive regulators of proliferation and differentiation for chondrocyte and cartilage proteoglycan synthesis is stimulated by insulin-like growth factor-1 (IGF-1). Transforming growth factor beta 1 (TGF-β1) modulates cell proliferation and enhances the deposition of ECM. Bone morphogenetic proteins (BMP-2) is a member of the TGF-β superfamily of proteins, it is expressed in the growth plate and regulates growth plate chondrogenesis by inducing chondrocyte proliferation and hypertrophy. These growth factors correlate with the cellular differentiation and increase the synthesis and deposition of ECM components by chondrocytes. **[18]**

Additionally, agarose is an attractive immobilization material because it is nontoxic, and gelation occurs under mild conditions. But cells are almost completely unable to attach because of a lack of a peptide for cell adhesion **[19]**. Furthermore, it has poor biodegradability; this property and the absence of appropriate enzymatic degradation systems in mammalian tissues **[6]**.

Consequently, the aim of this thesis is development and investigation of cartilage engineering. We try to fabricated fibrin + PRF scaffold in order to be able to enhance chondrocyte proliferation, differentiation and promote cartilage-specific ECM synthesis.more effective than only fibrin and agarose scaffold.

In the future, we can use the PRF biomaterial system to let the stem cells develop in the 3D fibrin that mixes with PRF scaffold scaffold. We have chosen to focus our attention in the current study on the effects of this carrier system on chondrogenic differentiation of MSCs **[19]**. A potential therapy to enhance healing of bone tissue is to deliver isolated mesenchymal stem cells (MSCs) to the site of a lesion to promote bone formation **[20]**. It may be effective to use for OA repair by cartilage tissue engineering.

Materials and methods

Cell culture

A human chondrosarcoma cell line, SW-1353 , were purchased from ATCC (ATCC, USA) and were inoculated in 60mm diameter dish and cultured in Leibovitz's L-15 medium containing 10% fetal bovine serum (Gibco, USA) , 1% penicillin-streptomycin solution and 1% L-glutamine (Sigma Chemical, St. Louis, MO). The cell line is cultured in an atmosphere of 37°C without CO_2.

Primary human chondrocyte culture

Osteoarthritis knee cartilage was obtained from patients undergoing total joint replacement surgery. Cartilage slices were cut into pieces (2~3 mm^3), and chondrocytes were released from articular cartilage by sequential enzymatic digestion with 1 mg/ml hyaluronidase (Sigma Chemical, St. Louis, MO) for 15 min, with 0.25% pronase for 30 min, and with 2mg/ml type Ⅱ collegenase (Sigma Chemical) for 12 h at 37 ℃ in Dulbecco's modified Eagles's medium (DMEM) containing antibiotics (100 U/ml penicillin, 100 U/ml streptomycin, and 2.5 mg/ml amphotericin B). After filtration through a 100-meshnylon mesh and centrifugation, chondrocyte residues were washed and seeded at a high density in DMEM supplemented with 10% fetal bovine serum (Gibcibrl) and antibiotics, and incubated at 37 ℃ in a humidified atmosphere of 5% CO_2 and 95% air .

Preparation of fibrin scaffolds

Bovine fibrin gel

Fibrin is made from bovine fibrinogen and thrombine. The fibrinogen (10 mg/ml, 0.5ml) (Sigma Chemical, St. Louis, MO) is dissolved in L-15 (no phenol red) and filtered with 0.22 -μm low protein binding filter. The fibrinogen solution is poured

into each well of a 24 multi-wells culture plate. Then, thrombin solution (5U /ml, 0.5ml) (Sigma Chemical, St. Louis, MO) is poured into each well to set a fibrin gel.

Human platelet-rich fibrin (PRF) gel

This technique requires neither anticoagulant nor bovine thrombin (nor any other gelling agent). It is nothing more than centrifuged blood without any addition. A blood sample is taken without anticoagulant in 10-mL tubes which are immediately centrifuged at 3000 rpm (approximately 400g according to our calculations) for 10 minutes. Fibrinogen is initially concentrated in the high part of the tube, before the circulating thrombin transforms it into fibrin. A fibrin clot is then obtained in the middle of the tube, just between the red corpuscles at the bottom and acellular plasma at the top.

Bovine fibrin sponge

Fibrin sponges are fabricated by conventional freeze-drying method. The bovine fibrin gel is frozen at -80^{o}C overnight, follows by freeze-drying under 6 Pa vacuum conditions for 24 hour to obtain a fibrin sponge. The diameter and thickness of sponge are measured.

Human platelet-rich fibrin sponge

The method is the same as the bovine fibrin sponges. They are fabricated by the conventional freeze-drying method, and the pore size compares with bovine fibrin sponges.

Seeding chondrocyte in 3D fibrin gels

The chondrocytes (second passages) are collected in L-15 with 10% FBS medium at 3 x 10^5 cells/ml, then mix with 0.5ml fibrinogen solution. A total of 0.5ml of thrombin solution is then mix with chondrocytes-fibrinogen at avolume ratio of 1:1 in each well. The final concentration of fibrinogen, thrombin, and chondrocytes are

10mg/ml, 5U/ml, and 1.5 x 10^5 cells/ml. After gelation completes, the new formed fibrin gels add 0.2ml chondrocyte culture medium. Cultures are carried out in a gassed incubator at 37^{o}C with no CO_2.

Seeding chondrocyte in 3D fibrin gel mixed with PRF exudates

See above the method of fibrin gel form. The fibrinogen solution mixed with chondrocytes first. And then added PRF exudates and thrombin solution. Finally, the 3D fibrin gel mixed with PRF exudates was produced.

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Quantify the concentration of the growth factors by ELISA kit

Sample collection and storage

Serum – use a serum separator tube (SST) and allowed sample to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000xg.

Plasma – collet plasma using EDTA as an anticoagulant. Centrifuge for 15 minutes at 1000xg within 30 minutes of collection. Assay immediately or aliquot and store samples at 2 - 8 ^{o}C for up to 4 weeks or freeze at -20 ^{o}C.

Fibrin – fibrin gel degradation product fibrin solution.

PRF – PRF gel exudates from blood releases exudates.

Platelet-derived growth factor-BB (PDGF-BB)

Prepare all reagents, standards, and samples as instructed. Add 100µl of Assay Diluent RD1X to each well. Then add 100µl Standard, control, and PRF gel released solution to each 96-well microplate, incubate 2 hours in room temperature. Aspirate and wash 4 times and add 200µl conjugate to each well. (serum / plasma samples incubate 2 hours in room temperature ; cell culture supernate samples incubate 1.5 hours in room temperature). Then aspirate and wash 4 times again. Add 200µl Substrate Solution to each well, incubate 30 minute in room temperature (protect from

light). Finally, add 50µl Stop Solution to each well. Read at 450 nm within 30 min (λ correction 540 or 570 nm) (R&D system) by ELISA reader (Hyperion).

Transforming growth factor beta 1 (TGF-β1)

Prepare all reagents, standards, and samples. Add 50µl of Assay Diluent RD 1-73 (for serum / plasma / fibrin / PRF) to each 96-well microplate . Then add 50µl Standard, control, fibrin and PRF gel released solution per well. Incubate for 2 hour at room temperature. Aspirate and wash 4 times and add 100µl of conjugate to each well, incubate for 2 hours at room temperature. Repeat the aspiration/wash step. Add 100 µl of substrate solution to each well and incubate for 30 minutes at room temperature. Protect from light. Add 100µl Stop Solution to each well. Read at 450 nm (R&D system).

Insulin-like growth factor-1 (IGF-1) :

See above description (R&D system).

Bone morphogenetic proteins (BMP-2) :

See above description (R&D system).

Morphological observation

Two types of fibrin sponge

The fibrin sponge is cut with a razor blade. The cross section of sponges is coated with gold. The inner microstructure of fibrin sponges is observed by a scanning electron microscope (SEM , Hitachi S-2400) at a voltage of 15 kV.

Chondrocyte proliferation and expansion

Chondrocyte are cultured in 2D & 3D fibrin, fibrin + PRF scaffold and in 2D & 3D agarose scaffold. Morphological observations of cells entrap within the gel are preformed with a confocal microscope every 4 days up to 12 days.

MTT assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide, a tetrazole) (Sigma Chemical, St. Louis, MO) is reduced to purple formazan in the mitochondria of living cells. MTT stock solution was used 5mg/ml MTT in RPMI-1640 without phenol red. MSCs cultured in 3D gel complexes were incubated with 500λ of Trypsin-EDTA at 37^{o}C for 30 min. The cells were collected and washed with PBS solution. Added MTT working solution into wells being assayed, 250λfor each well of 24-well plate, and incubated approximation 4 hours at 37^{o}C in dark. Then, added 200μl DMSO (Sigma Chemical, St. Louis, MO), 100μl of supernatant was removed and transferred to a 96-well microplate. Absorbance was measured on an ELISA plate reader at a wavelength of 540 nm.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

Cells were collected from each target experiment on 4, 8 and 12 days. For evaluating the expression of mRNA level of aggrecan and type II collagen, total RNA was isolated from samples for time courses. In brief, complementary DNA was synthesized in a 25-μl reaction mixture containing 5 μg of total RNA, 2.5 mM of each dNTP, 1mM of random hexamer primers, and 10U of M-MLV reverse transcriptase (Epicentre, Madison, WI), by incubation at 37℃ for 90 min. The resulting cDNA (2μl) was subjected to PCR using Taq DNA polymerase (Epicentre) and specific primers for type II collagen, aggrecan and GAPDH as shown in Table Ⅰ. For type II collagen and aggrecan, the PCR protocol was 40 cycles at 94℃ for 1 min, 60℃ for 1 min, and 72 ℃ for 1 min. In each experiment, amplification of cDNA for the housekeeping gene, GAPDH (E-oligo), was used as an internal standard. PCR products were analyzed on 1.5% agarose (Amresco, Solon, OH) gels.

Histological analysis

Haematoxylin and Eosin stain (H&E stain)

1. Bring sections to distilled water.

2. Stain nuclei with the alum haematoxylin.

3. Rinse in running tap water.

4. Differentiate with 0.3% acid alcohol.

5. Rinse in running tap water.

6. Rinse in Scott's tap water substitute.

7. Rinse in tap water.

8. Stain with eosin 2 mins.

9. Dehydrate, clear and mount.

Alcian blue stain

1. Deparaffinize slides and hydrate to distilled water.

2. Stain in alcian blue solution for 30 minutes.

3. Wash in running tap water for 2 minutes.

4. Rinse in distilled water.

5. Counterstain in nuclear fast red solution for 5 minutes.

6. Wash in running tap water for 1 minute.

7. Dehydrate and through 95% alcohol, 2 changes of absolute alcohol, 3 minutes each.

8. Clear in xylene substitute.

9. Mount with resinous mounting medium.

Periodic acid-Schiff stain (PAS stain)

1. Deparaffinize and hydrate to water.

2. Oxidize in 0.5% periodic acid solution for 5 minutes.

3. Rinse in distilled water.

4. Place in Schiff reagent for 15 minutes (Sections become light pink color during

this step).

5. Wash in lukewarm tap water for 5 minutes (Immediately sections turn dark pink Color).
6. Counterstain in Mayer's hematoxylin for 1 minute**.**
7. Wash in tap water for 5 minutes.
8. Dehydrate and coverslip using a synthetic mounting medium.

Injectable using fibrin and fibrin combined PRF gel in vivo

Prepared fibrinogen (10mg/ml, 500λ), thrombin (5U/ml, 500λ) solution and PRF exudates 50 λ to produce fibrin gel and fibrin + PRF gel. Two types of scaffolds are including 3 x 10^6 cells/ml. Prepared 1ml mixture inject into nude mice by syringe. Then sacrifice after 4 days, 8 days. Stained with H&E stain and Alcian blue. Images were acquired with a light microscope.

Statistical analysis

Comparisons for MTT assay for chindrocytes and SW-1353 survival and RT-PCR for quantitative expression level of aggrecan and type II collagen in fibrin and fibrin+PRF scaffold. Data were analyzed using independent samples t-tests. All data are expressed as means ± standard deviations (SD) of three independent experiments and $p< 0.05$.

Results

The production of platelet-rich fibrin (PRF) according to the Process protocol.

The blood sample was taken without anticoagulant and any addition in 10-ml tube which were immediately centrifuged at 3000 rpm for about 10 minutes. After centrifuged, at the top of tube was acellular plasma, the fibrin clot, PRF, was obtained in the middle of the tube, and red corpuscles were at the bottom (Figure 1 left). The PRF fibrin clot was obtained into three parts: buffy columns corresponding to platelet accumulation that trapped into the PRF fibrin matrix between red thrombus in contact with red blood corpules and an acellluar fibrin gel (Figure 1 right).

Cytokines and growth factors quantification of blood-derivative products compared with PRF exudates.

The concentration of PDGF-BB, IGF-1, TGF-β1, and BMP-2 were quantitated by ELISA kit. These cytokines and growth factors value of PRF exudates were higher than blood-derivative products (serum, plasma, fibrin) averagely, excepted TGF-β1. The fibrin had the lowest volume of IGF-1 and TGF-β1 concentration. Plasma had the highest value of TGF-β1 concentration (Figure 2A,B,C,D).

The comparison of fibrin and PRF sponge by freeze-drying and the inner construct by scanning electronic microscopy (SEM).

By freeze-drying method, the fibrin and PRF gel were form sponges (Figure 3A). The cross-sectional SEM photographs showed that two types of sponge scaffold possessed an interconnected porous structure with an average pore size about 40μm. It could provide the adaptive internal pore to let cells develop (Figure 3B).

The defect of fibrin and PRF sponges.

Fibrin and PRF sponges were appropriate to be scaffolds. But when solution dropped into the fibrin and PRF sponge, the structure was destroyed quickly (Figure 4). Because of the imperfection we chose the fibrin gel form to be the scaffold for developing cells finally.

The two-dimension (2D) and three- dimension (3D) scaffold model.

Coding agarose gel around per well prevented cell adhesion. Then made scaffold by using three kinds of materials, fibrin, fibrin + PRF and agarose. PRF clots placed for a short time about five minutes, the PRF exudates (Figure 5 left) were mixed with fibrinogen and then added thrombin to form the fibrin + PRF gel. 2D scaffold was indicated that cells were cultivated on the surface of the gel (Figure 2 the upper right). And 3D scaffold was that cells were mixed with fibrinogen before thrombin added (Figure 5 the lower right).

Morphology of fibrin and fibrin + PRF gel for time courses.

Cells developed in fibrin and fibrin + PRF gel, the structure degraded by degree during 1-12 days. On the twelfth day, the fibrin and fibrin + PRF gel morphology started to become turbid. According to previously**,** coarse gels were made of thicker fibers due to lateral aggregation of protofibrils, which resulted in turbid and lost gels with larger pore size **[12]**. About two weeks, the gel was complete degraded. It indicated that the gel could maintain its fine condition about 12 days (Figure 6).

Morphology of SW-1353 cells and primary human chondrocytes growth on 2D fibrin and fibrin + PRF scaffold.

Morphology of primary human chondrocytes (Figure 7). The SW-1353 cells and

primary chondrocytes seeded not only in fibrin gel but also in fibrin + PRF gel that cell number increased observably during 1,4,8,12 days (Figure 8A-D). In agarose scaffold, cells were almost completely unable to attach (data not show).

Cell growth rate of SW-1353 cells on 2D and in 3D fibrin, fibrin + PRF, and agarose scaffold by MTT assay.

The SW-1353 cells and chondrocytes distributed within 2D and 3D fibrin, fibrin + PRF, and agarose gel scaffold for 4,8,12 days. The cell growth rate for fibrin and fibrin + PRF were increased evidently. Furthermore, the cell number of fibrin + PRF scaffold was more effective increased than only fibrin. Contrariously, the cell growth rate of agarose scaffold was almost changeless, even decreased (Figure 9A, B).

Expression level of type II collagen and aggrecan for chondrocytes seeded in fibrin and fibrin + PRF gel scaffold during time courses.

After 4, 8, and 12 days, compared with chondrocytes that seeded in fibrin, mRNA expression of type II collagen and aggrecan for chondrocytes seeded in fibrin + PRF scaffold was obvious up-regulated. This result demonstrated that in fibrin + PRF three-dimension tissue culture of expanded articular chondrocytes re-differentiation effect better than chondrocytes just seeded in fibrin (Figure 10A, B).

Histological analysis of chondrocytes in 3D fibrin and fibrin+PRF scaffold in vitro.

Chondrocytes volume in fibrin + PRF gel were better than in fibrin gel scaffold after 8 days by H&E staining (Figure 11A). GAGs accumulation of chondrocytes in fibrin + PRF gel were more than in fibrin gel scaffold after 8 days by Alcian blue staining (Figure 11B). Proteoglycan accumulation of chondrocytes in fibrin + PRF gel

was more than in gel fibrin scaffold after 8 days by PAS staining (Figure 11C). Because of it deposited higher amounts GAGs and proteoglycan in fibrin + PRF gel than in fibrin gel scaffold. It was meat chondrocytes re-differentiation significantly in fibrin by PRF incorporation gel scaffold

Discussion

Healthy articular cartilage is composed of chondrocytes in ECM. Chondrocytes secret such ECM molecules as type II collagen and GAGs to keep the mechanical properties of the tissue. Cartilage is aneural and avascular and therefore has alimited capacity for self-repair. Classic methods of cartilage replaced such as autographing, allografing and the use of synthetic materials are not ideal **[2]**. The goal of cartilage engineering is proposed that provides a cartilaginous constructs to replace abnormal cartilage, because it is able to overcome problems associated with tradition cartilage replacement methods.

It is definite that fibrin-based biomaterials possess several features to make an important role of the scaffold material for cell proliferation and differentiation. Fibrin gel improves seeding capacity of the scaffold, supports equal distribution of cells and stimulates higher chondrogenic phenotype expression **[26]**. But the growth factors and cytokines of fibrin are limited. Because of platelet-rich fibrin (PRF) has abundant growth factors and cytokines more than other blood-derivative products, just like serum, plasma, and fibrin. The platelet cytokines, IGF-1, TGF-β1, and PDGF-BB are the potent stimulators of chobdrocytes proliferation. Furthermore, BMP-2, it can provide chondrocytes used for preparing tissue engineered cartilage repair to maintain of a differentiated phenotype. If we could use a simple and easy method to generate a new type scaffold that fibrin gel incorporates the extra materials - PRF including a plenty of growth factors and cytokines, it might improve chondrocytes seeding efficiency and expression of cartilage specific gene. In this thesis, our aim is that establish a biodegradation fibrin + PRF scaffold which can permit the cells to retain their native phenotype and produce their extracellular components.

Cartilage engineering approaches use either microporous hydrogels or macroporous

sponge-like solid polymeric scaffolds in common. However, fibrin is mechanically too weak to maintain the desired shapes and structures **[21]**. When we made the fibrin and PRF sponge via freeze-drying, this 3D structure was friable and destroyed quickly by dropping solution. The fibrin and PRF sponges were too weak to hold the three-dimension structure. So using gel form took place of sponge. An characteristic of fibrin is an increasing instability and solubility over time, due to fibrinolysis. Rapid degradation could be an advantage in wound sealing or other surgical applications as well as for cell and growth factor delivery **[12]**. Obtained fibrin and fibrin + PRF gel morphology, they could maintain their condition about two weeks (Fig.6). Transparency is an additional favourable characteristic as it allows observation of the single cells during the early culture period **[12]**. During 1-12days, the gel was turbid gradually. It meant that gel became coarose, which result in turbid. The term stable fibrin and fibrin + PRF gel scaffold could let chondrocytes steady growth.

Platelet-rich fibrin (PRF) is a new generation of platelet concentrates via simplified preparation without biochemical blood handling. Initial analyses revealed that the absence of anti-coagulant in the collection tube necessary induced massive platelet activation **[13]**. Slow fibrin polymerization during PRF processing leads to the intrinsic incorporation of platelet cytokines and glycanic chains in the fibrin meshes **[16]**. So when chondrocytes developed in fibrin + PRF scaffold, the cell growth rate was significantly increased superior to only fibrin or agarose scaffold (Fig.8、9). In vitro SW-1353 cells and primary human chondrocytes seeded in fibrin + PRF gel scaffold that shows the available for type II collagen and GAGs mRNA expression better than in fibrin scaffold (Fig.10). Histologically, the chondrocytes bred in 3D fibrin + PRF gel scaffold that the GAGs and proteoglycan accumulation much more than in 3D fibrin alone. The results show that the fibrin + PRF scaffold enhance

chondrogenetic proliferation, differentiation and promote cartilage-specific ECM synthesis. It is comfired such as PDGF-BB, TGF-β1, BMP-2 or IGF-1, to modulate the cellular differentiation and increase the synthesis and deposition of ECM components by chondrocytes **[29]**. After we will implant each scaffold into the backs of nude mice by subcutaneous implantation, the experiment is going now.

Chondrogenic differentiation of MSCs has been effected by the application of growth factors from the TGF-β superfamily **[28]**. Moreover, these growth factors and cytokines contain in PRF exudates could facilitate mesenchymal stem cells (MSCs) proceeding chondrogenic differentiation. The tissue-engineered construct in chodrogenic media exhibited elevated expression of glycosaminoglycan and chondrogenic marker genes **[27]**. So the fibrin + PRF gel might be the unique scaffold for stem cell-based cartilage repair.

The most important aspect of cartilage tissue engineering is the design of a scaffold structure that controls three-dimensional shape and guides tissue development **[26]**. The fibrin gel with PRF exudates incorporate to fabricate the compound scaffold using in cartilage tissue engineering that is a promising new type scaffold and have the potential to be processed for cartilage tissue repair.

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附圖

Experimental procedures :

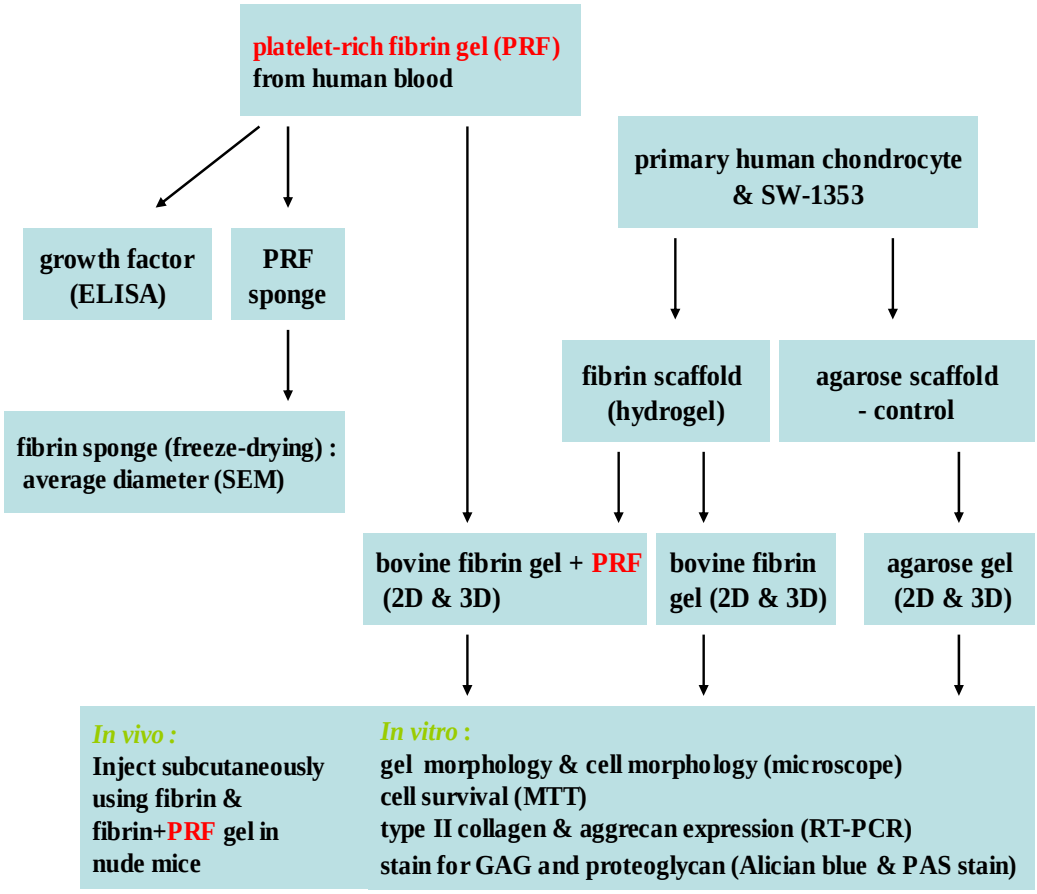


TABLE Ⅰ. Primer Sequences Used for the Reverse-Transcription Polymerase Chain Reaction

Primer sequences	Fragment size (bp)
Type II collagen	
Sense : 5'-AATTCGGTGTGGACATAGGG -3'	218
Antisence : 5'- TCTGCCCAGTTCAGGTCTCT-3'	
Aggrecan	
Sense : 5'-AGGAGTCCCTGACCTGGTTT -3'	171
Antisence : 5'-CCTGACAGATCTGCCTCTCC -3'	
GAPDH	
Sense : 5'- CAAGGCTGAGAACGGGAAGC-3'	195
Antisence : 5'- AGGGGGCAGAGATGATGACC-3'	

Fig.1

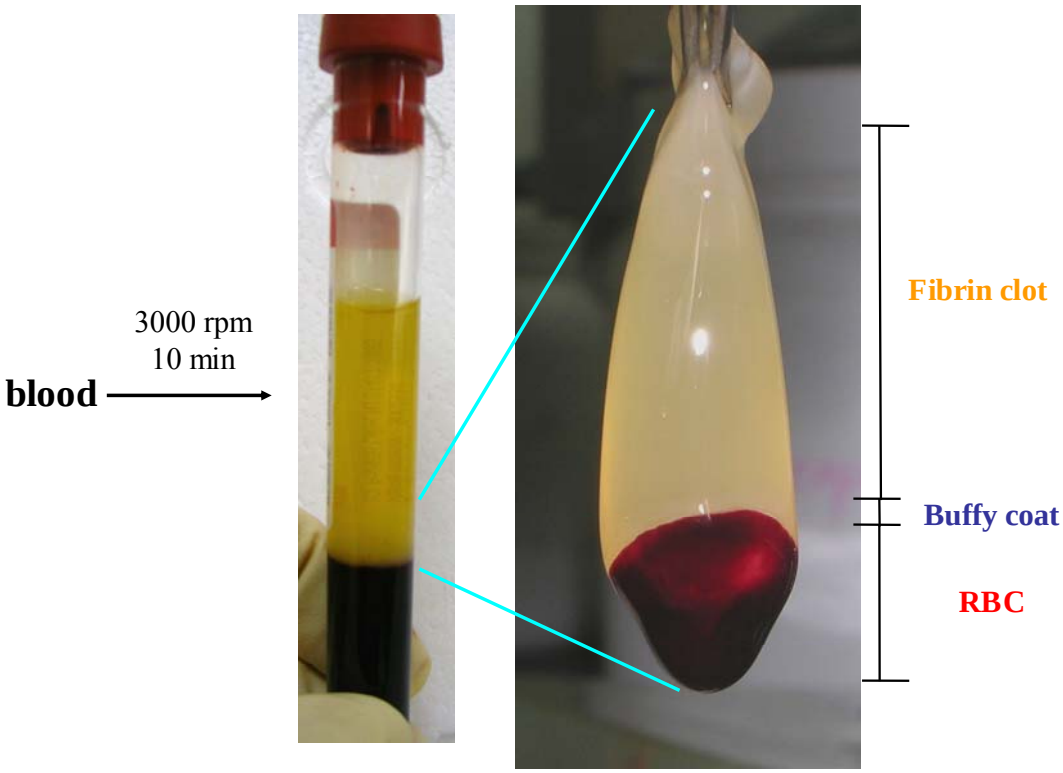


Fig.1 : Obtain the human platelet-rich fibrin (PRF) gel from human blood after centrifugation according to Process official protocol.

Human blood centrifugated immediately form the composition of PRF. The top to bottom of without anticoagulant tube are platelet-poor plasma (PPP), platelet-rich fibrin (PRF), and red corpuscules base. The composition of PRF are fibrin clot, buffy clot within the accumulation of platelet trapped into PRF matrix, and red thrombus.

Fig. 2A

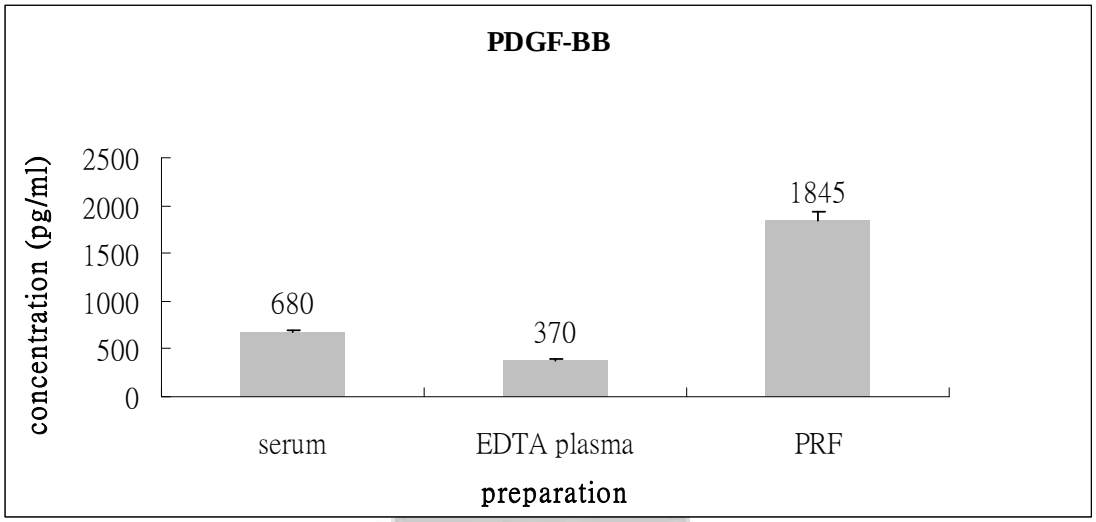

PDGF-BB
concentration (pg/ml)
2500
2000
1500
1000
500
0
680
370
1845
serum
EDTA plasma
PRF
preparation

Fig. 2B

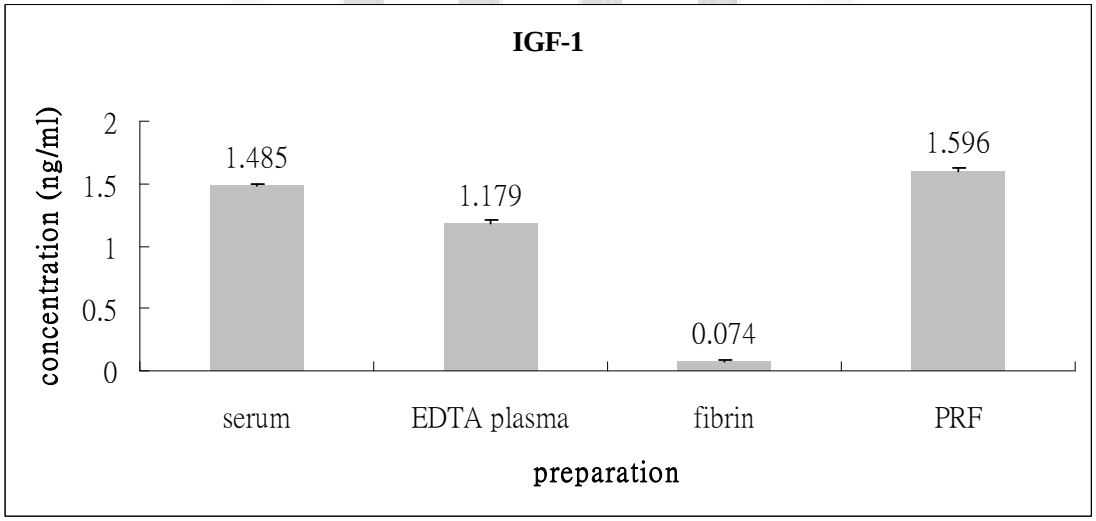

IGF-1
concentration (ng/ml)
2
1.5
1
0.5
0
1.485
1.179
0.074
1.596
serum
EDTA plasma
fibrin
PRF
preparation

Fig.2C

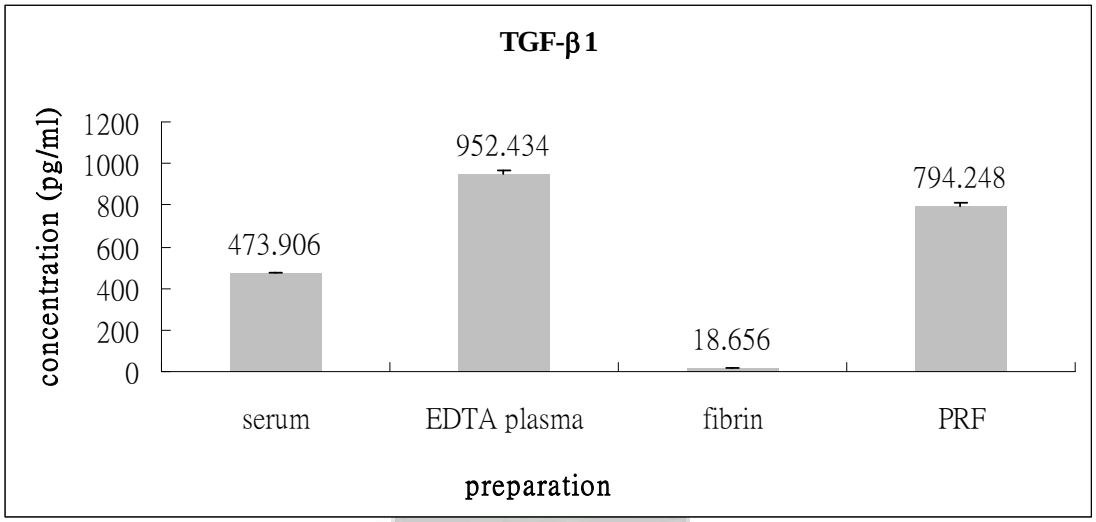

TGF-β1
concentration (pg/ml)
1200
1000
800
600
400
200
0
473.906
952.434
18.656
794.248
serum
EDTA plasma
fibrin
PRF
preparation

Fig.2D

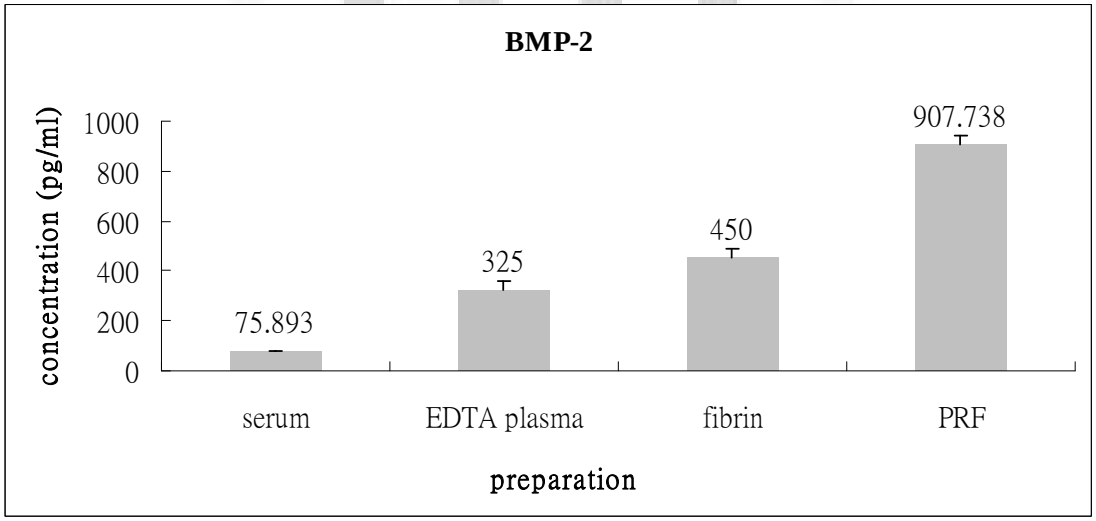

BMP-2
concentration (pg/ml)
1000
800
600
400
200
0
75.893
325
450
907.738
serum
EDTA plasma
fibrin
PRF
preparation

Fig.2 : Growth factors and cytokines ELISA quantification for human serum, plasma, fibrin and PRF exudates (n=3).

Collected serum by separator tube (SST) and allowed sample to clot for 30 minutes before centrifugation for 15 minutes at approximately 1000xg. Using EDTA anticoagulant tube collected plasma and centrifugated for 15 minutes at 1000xg within 30 minutes of collection. Got fibrin sample when fibrin gel degraded. PRF exudates were from PRF gel by blood centrifugation.

(A) PDGF-BB concentration quantification for serum, plasma, and PRF exudates.

(B) IGF-1 concentration quantification for serum, plasma, fibrin and PRF exudates.

(C) TGF-β1 concentration quantification for serum, plasma, fibrin and PRF exudates.

(D) BMP-2 concentration quantification for serum, plasma, fibrin and PRF exudates.

Fig.3A

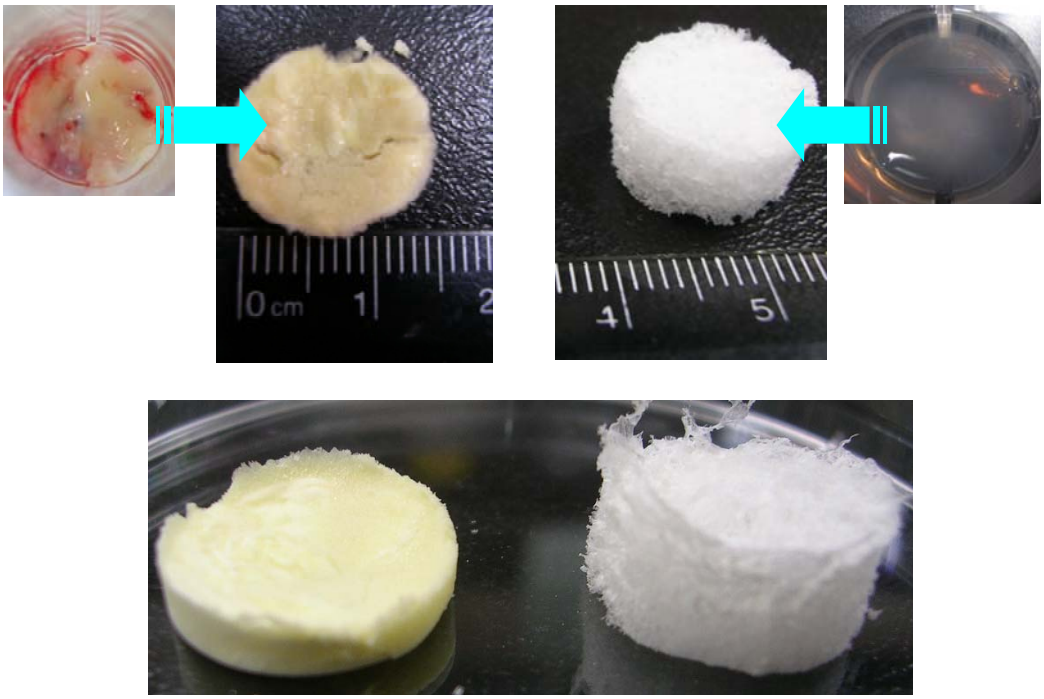


Fig.3B

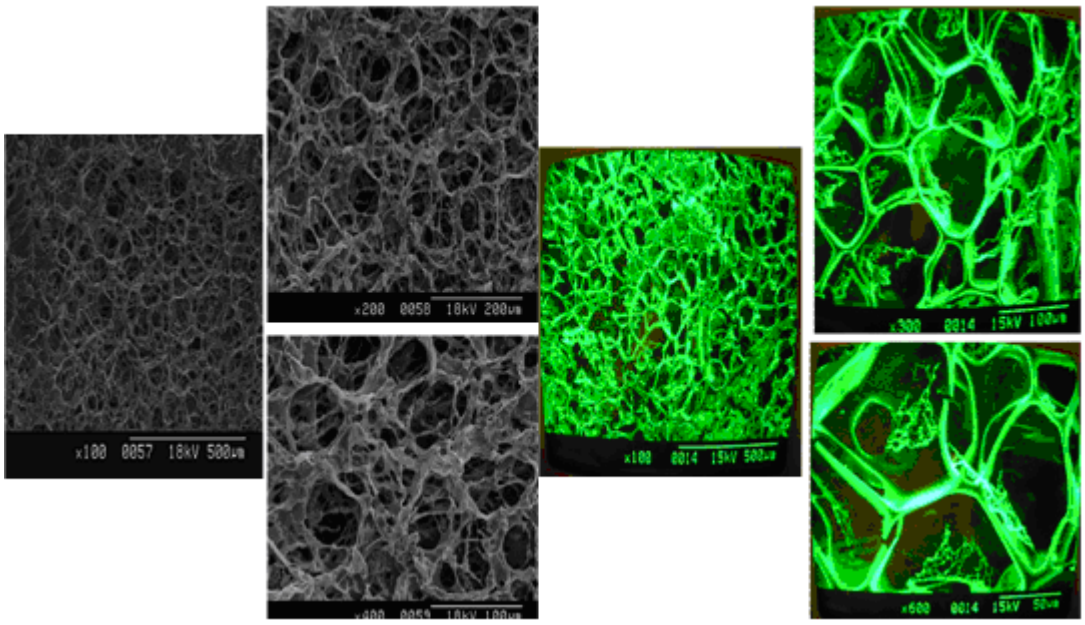


Fig.3 : Products of PRF sponge and fibrin sponge by freeze-drying and cross-section scanning electron morphology (SEM) photographs of their inner structure.

(A) The fibrinogen (10mg/ml, 500λ) mixed with thrombin (5U/ml, 500λ) to form the fibrin gel and blood centrifugation immediately with no anticoagulant tube form PRF . Then using conventional freeze-drying method fabricated the dehydrate fibrin and PRF sponges.

(B) Observation the inner structure of fibrin and PRF sponges by SEM. The internal pore structure of sponges were favose. And the pore size was available for chondrocytes growth in it.

Fig.4

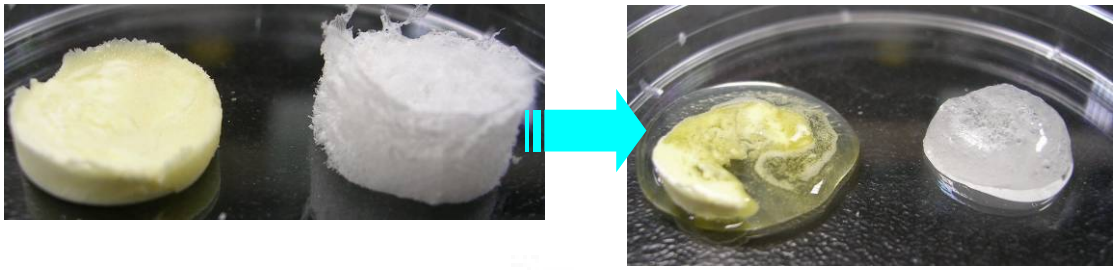


Fig.4 : Disadvantage of the PRF and fibrin sponge.

When the PRF and fibrin sponge trapped into solution, the 3D structures were destroyed. Because of the sponge substance was unstable to maintain its construction. It couldn't let cells seed in this form of scaffold.

Fig.5

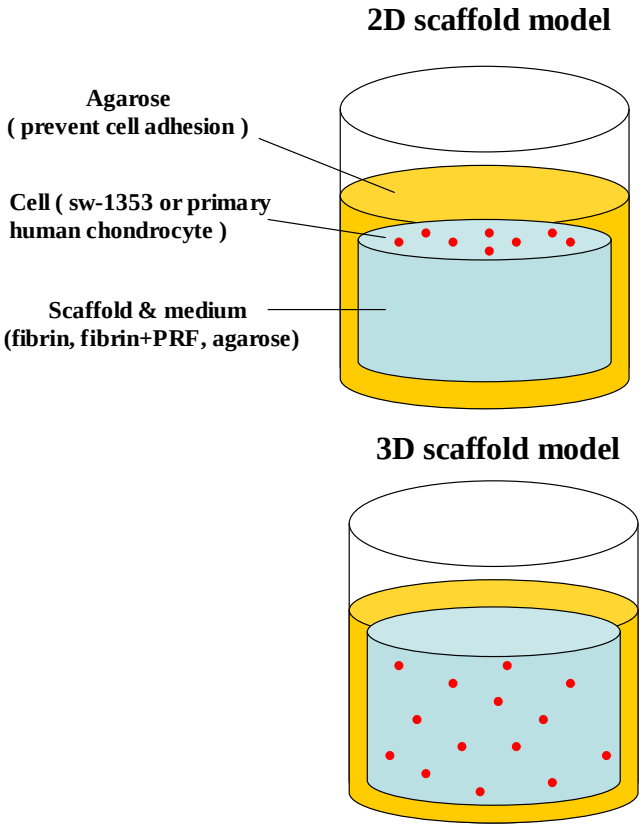

2D scaffold model
Agarose
(prevent cell adhesion)
Cell (sw-1353 or primary
human chondrocyte)
Scaffold & medium
(fibrin, fibrin+PRF, agarose)
3D scaffold model

Fig.5: The PRF gel releases solution and cells seeded on 2D and in 3D scaffold model (an individual well of a 24-well plate).

The three types of scaffold are fibrin gel, fibrin adding PRF exudates gel, amd agarose gel. Cells developed on the surface of scaffold to form the 2D model. The 3D model was cells mixed with fibrinogen or agarose solution before gel forming.

Fig. 6

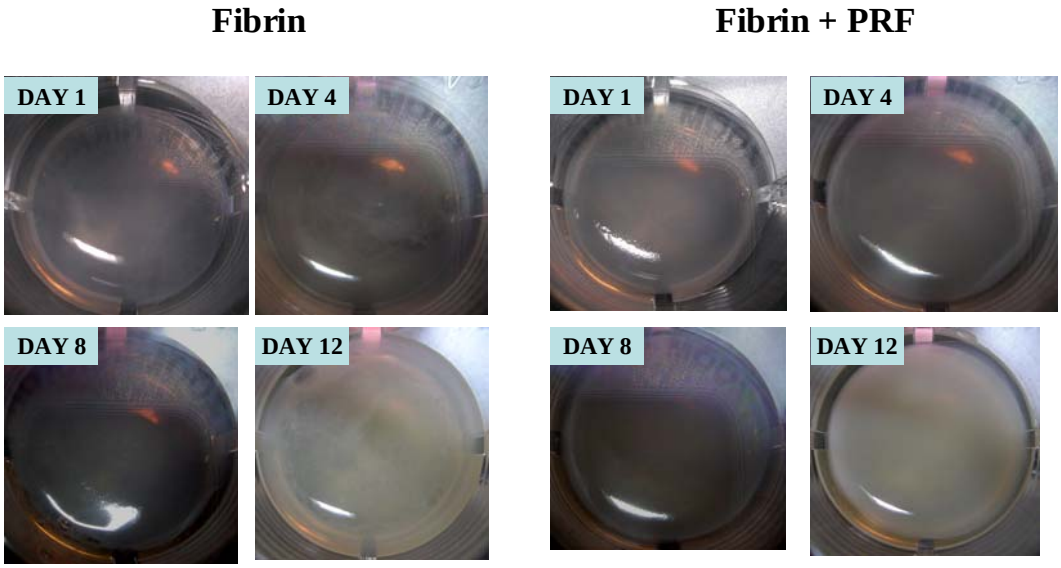


Fig.6 : Morphology of cells seeded in fibrin and fibrin+PRF gel for 1,4,8,12 days.

Fibrinogen (10mg/ml, 250λ), thrombin (5U/ml, 250λ), PRF exduate 50λ. SW-1353 cells and primary human chondrocytes seeded in fibrin and fibrin + PRF gel scaffold after 4,8,12 days. The fibrin and fibrin + PRF gel became turbid definitely on twelfth day.

Fig. 7

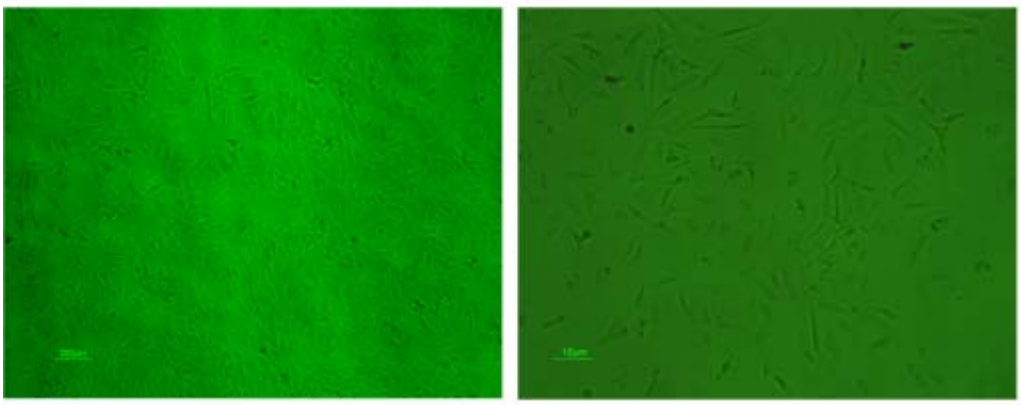


Fig.7 : Cell morphology of primary human chondrocytes (P1) using microscopy. (40x, 100x)

Fig. 8A

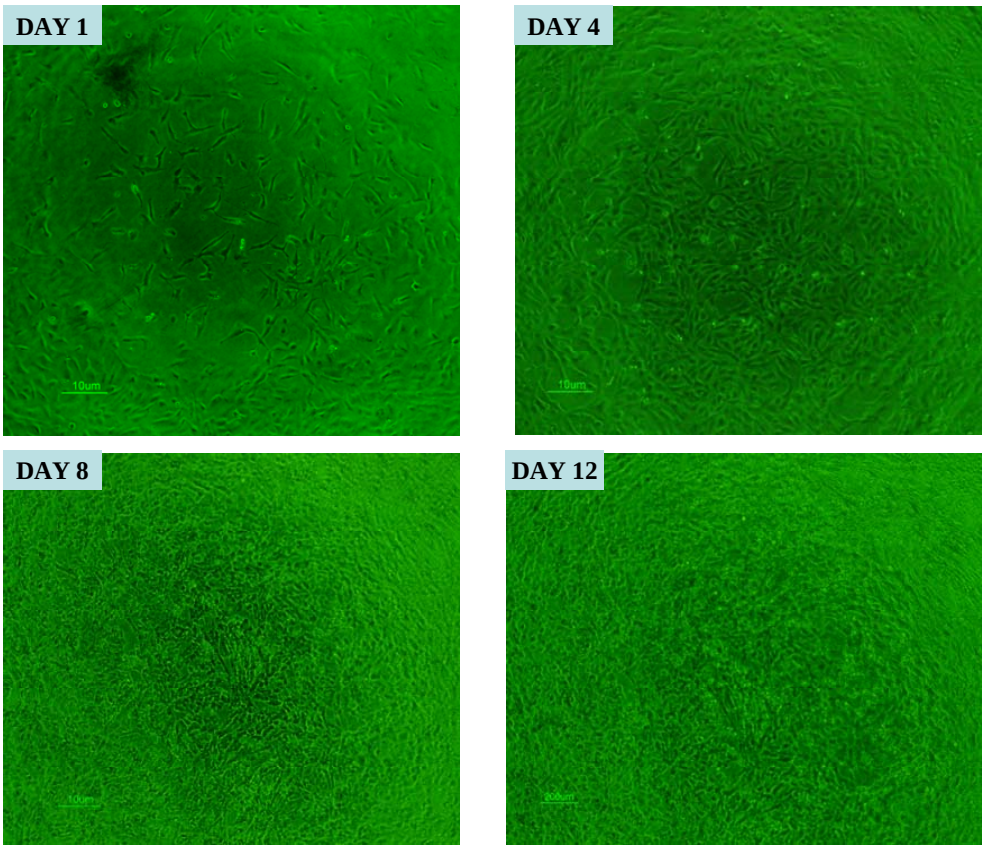


Fig. 8B

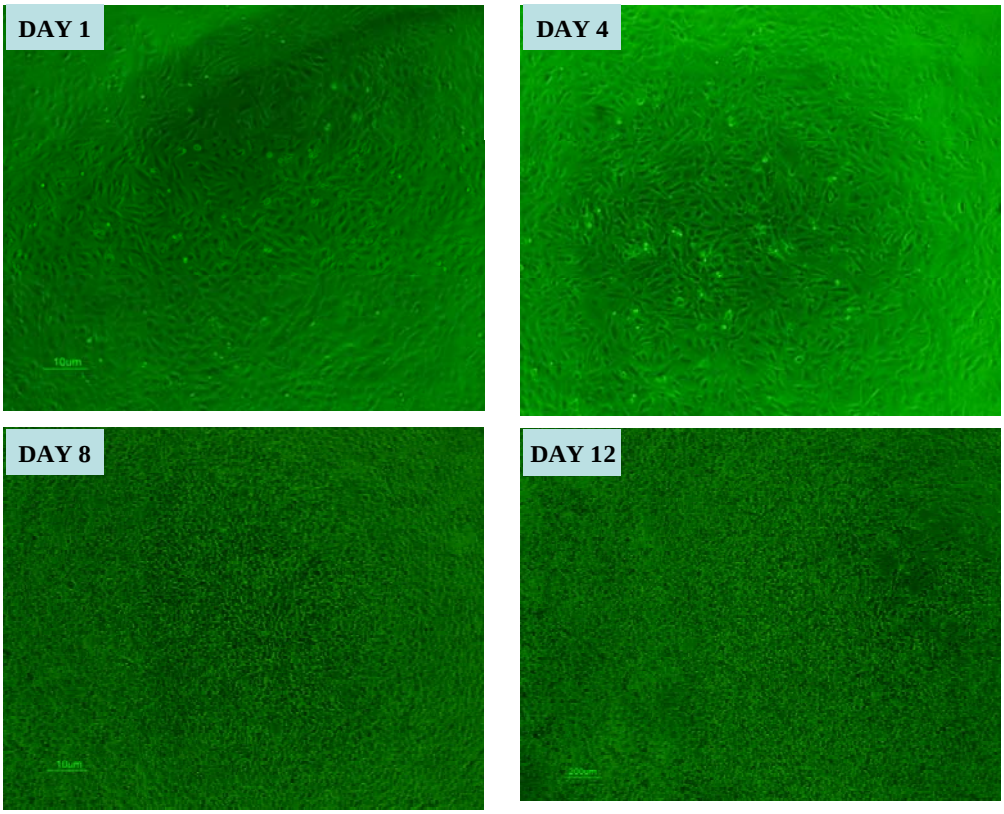


Fig. 8C

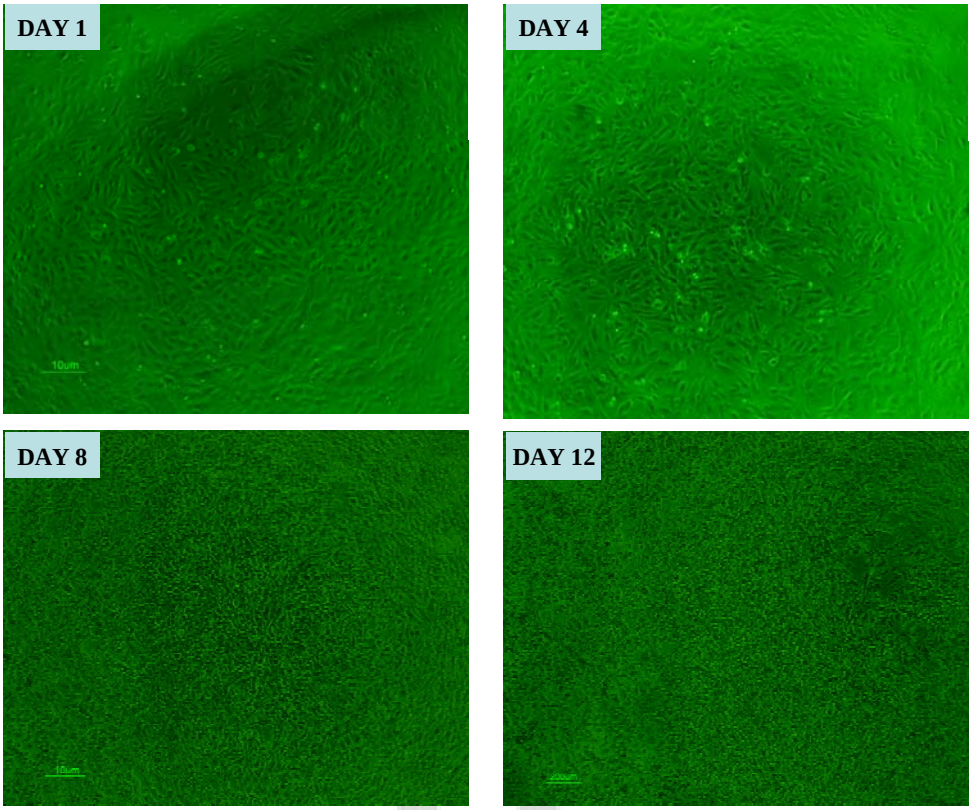


Fig. 8D

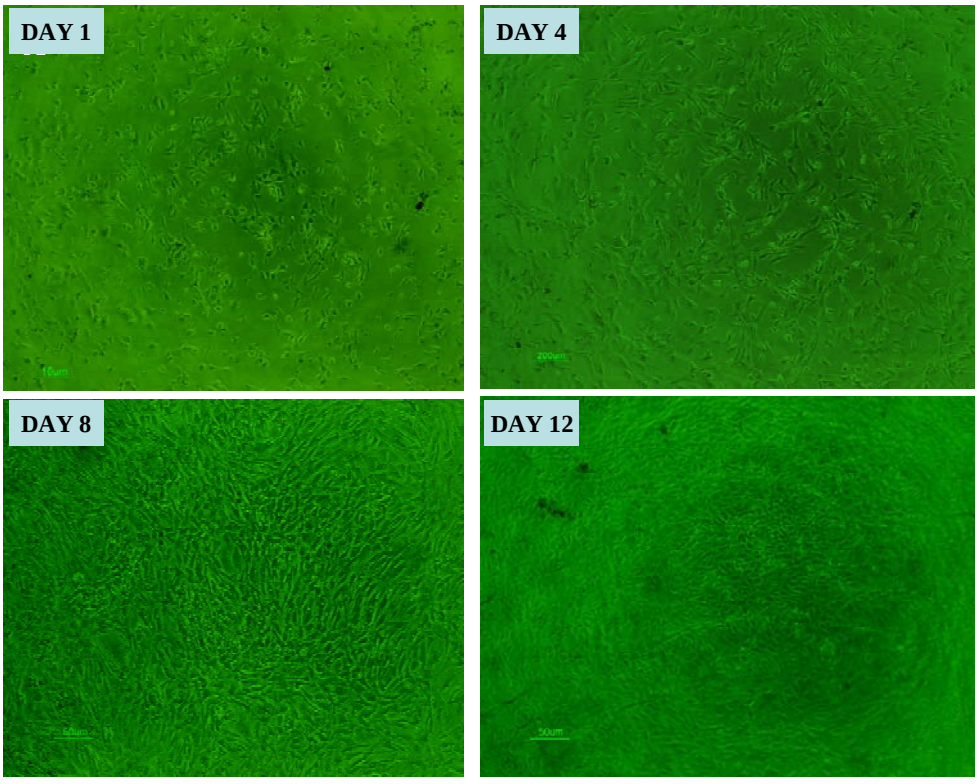


Fig. 8 : Morphology of SW-1353 cells and primary human chondrocytes viability cultured on 2D fibrin and fibrin + PRF scaffold after 1, 4, 8,12 days.

Cells cultivated on two kinds of 2D scaffold to evaluate the cells growth rate during time courses. Cells proliferation was higher in both fibrin and fibrin + PRF gels scaffold.

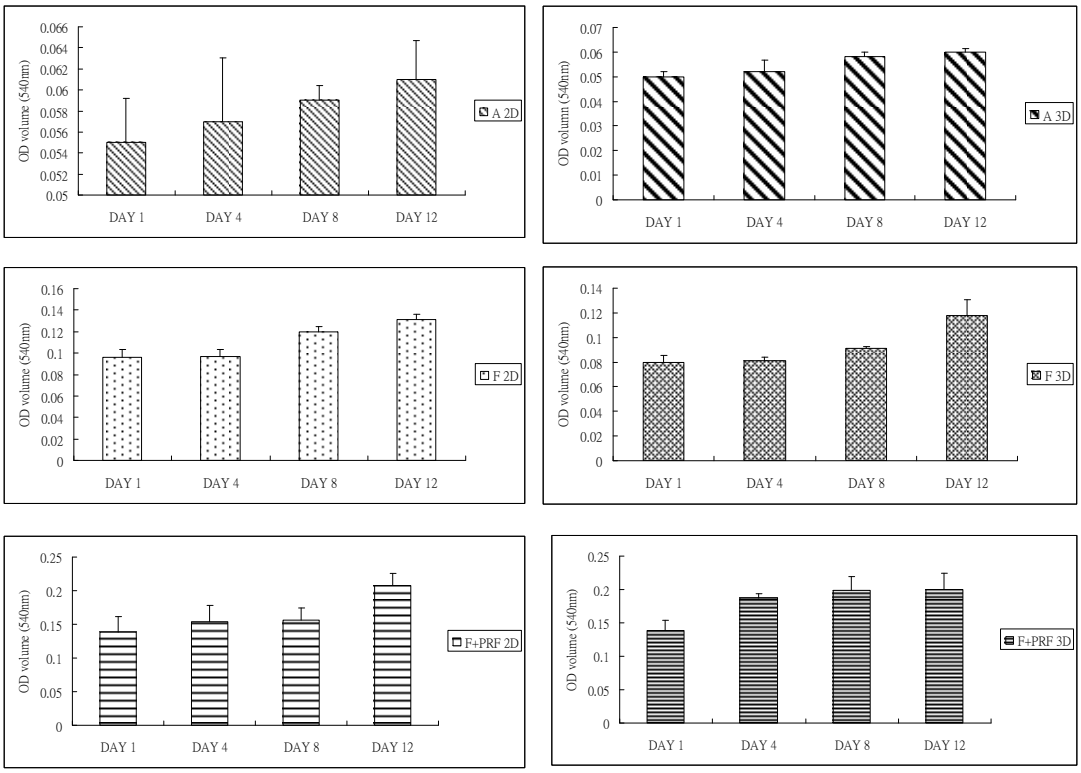
(A) SW-1353 cells seeded on 2D fibrin scaffold. Magnification, 40x.

(B) SW-1353 cells seeded on 2D fibrin + PRF scaffold. Magnification, 40x.

(C) Primary human chondrocytes seeded on 2D fibrin scaffold. Magnification, 40x.

(D) Primary human chondrocytes seeded on 2D fibrin + PRF scaffold. Magnification, 40x.

Fig. 9A


OD volume (540nm)
DAY 1
DAY 4
DAY 8
DAY 12
A 2D
A 3D
F 2D
F 3D
F+PRF 2D
F+PRF 3D

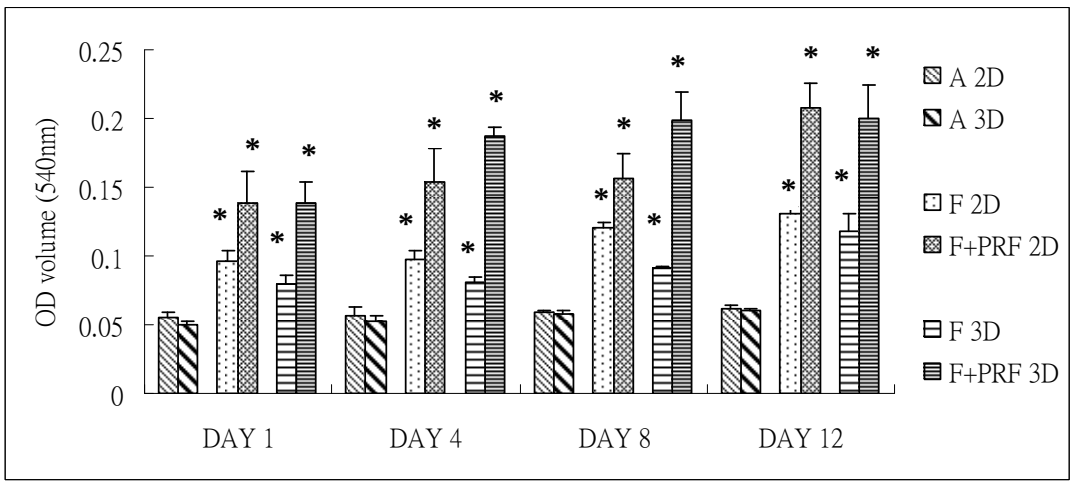
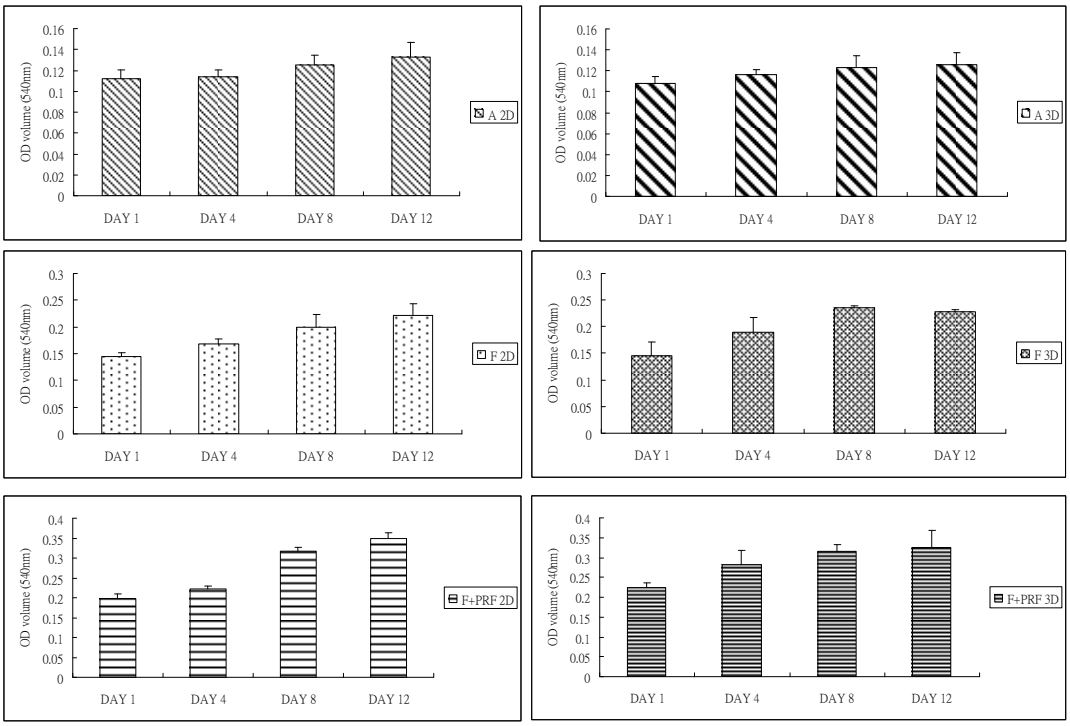

OD volume (540nm)
0.25
0.2
0.15
0.1
0.05
0
DAY 1
DAY 4
DAY 8
DAY 12
A 2D
A 3D
F 2D
F+PRF 2D
F 3D
F+PRF 3D

Fig. 9B


OD volume (540nm)
DAY 1
DAY 4
DAY 8
DAY 12
A 2D
A 3D
F 2D
F 3D
F+PRF 2D
F+PRF 3D

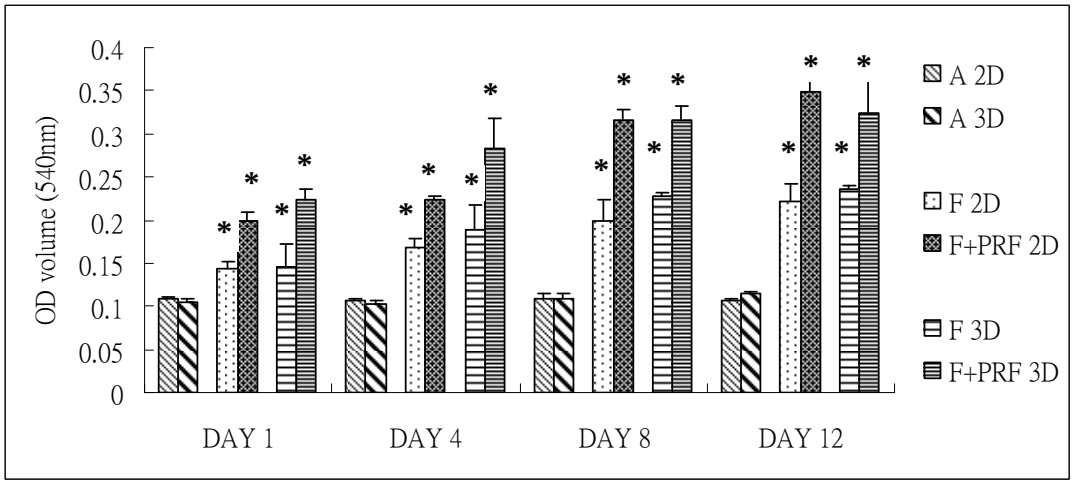

OD volume (540nm)
DAY 1
DAY 4
DAY 8
DAY 12
A 2D
A 3D
F 2D
F+PRF 2D
F 3D
F+PRF 3D

Fig. 9 : MTT OD value assay.

SW-1353 cells and chondrocytes cultured on 2D and in 3D fibrin, fibrin + PRF, and agarose scaffold for time courses. (n=3, *:$p<0.05$, fibrin and fibrin + PRF compared with the agarose, respectively) Significantly, chondrocytes proliferation was higher in both fibrin and fibrin + PRF gels versus 2D fibrin and fibrin + PRF gels.

(A) SW-1353 cells seeded on 2D and in 3D fibrin, fibrin + PRF, and agarose scaffold for 1, 4, 8, 12 days.

(B) Chondrocytes seeded on 2D and in 3D fibrin, fibrin + PRF, and agarose scaffold. for 1, 4, 8, 12 days.

Fig. 10A

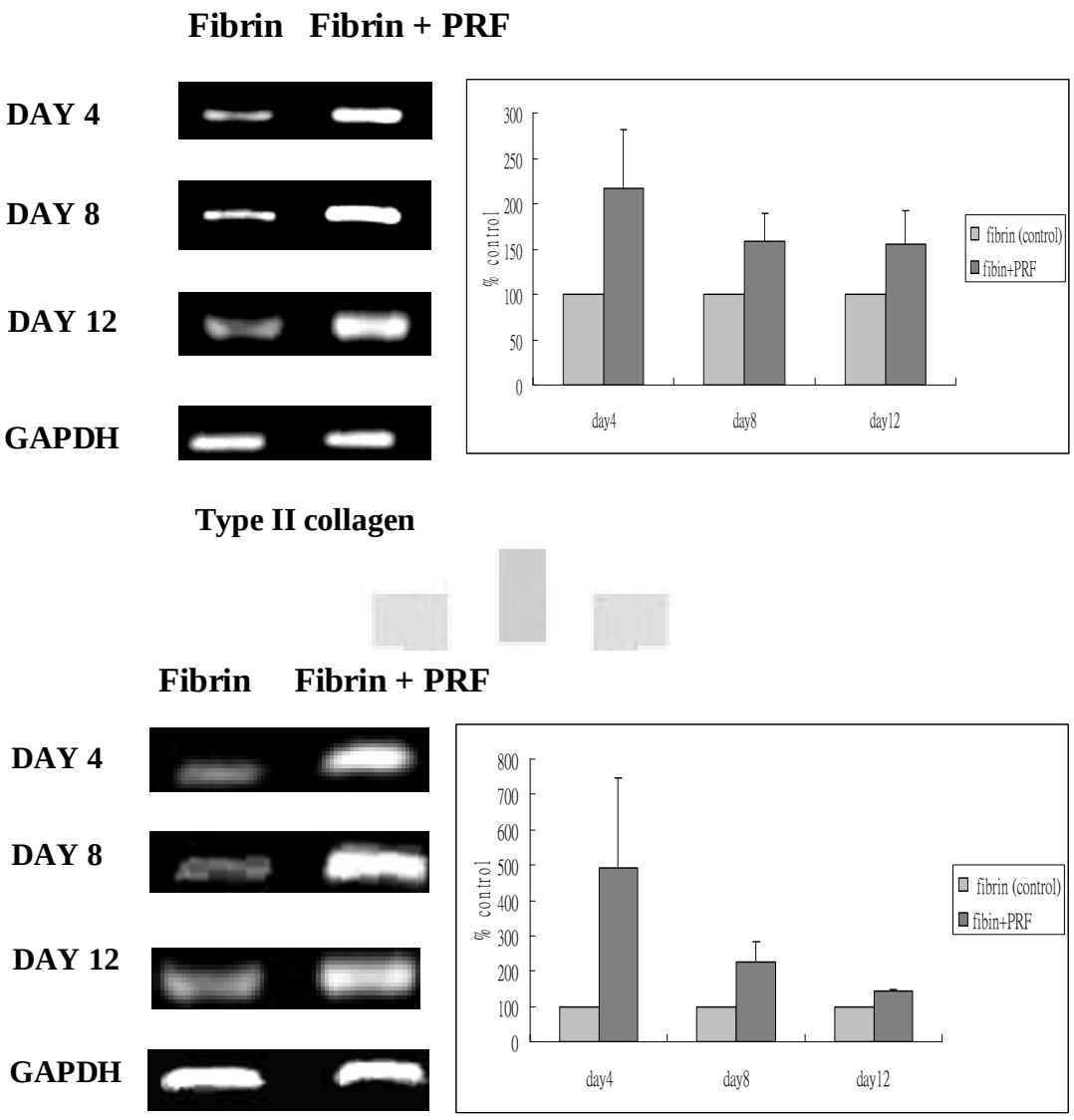

Fibrin
Fibrin + PRF
DAY 4
DAY 8
DAY 12
GAPDH
% control
300
250
200
150
100
50
0
day4
day8
day12
fibrin (control)
fibin+PRF
Type II collagen
Fibrin
Fibrin + PRF
DAY 4
DAY 8
DAY 12
GAPDH
% control
800
700
600
500
400
300
200
100
0
day4
day8
day12
fibrin (control)
fibin+PRF
Aggrecan

Fig. 10B

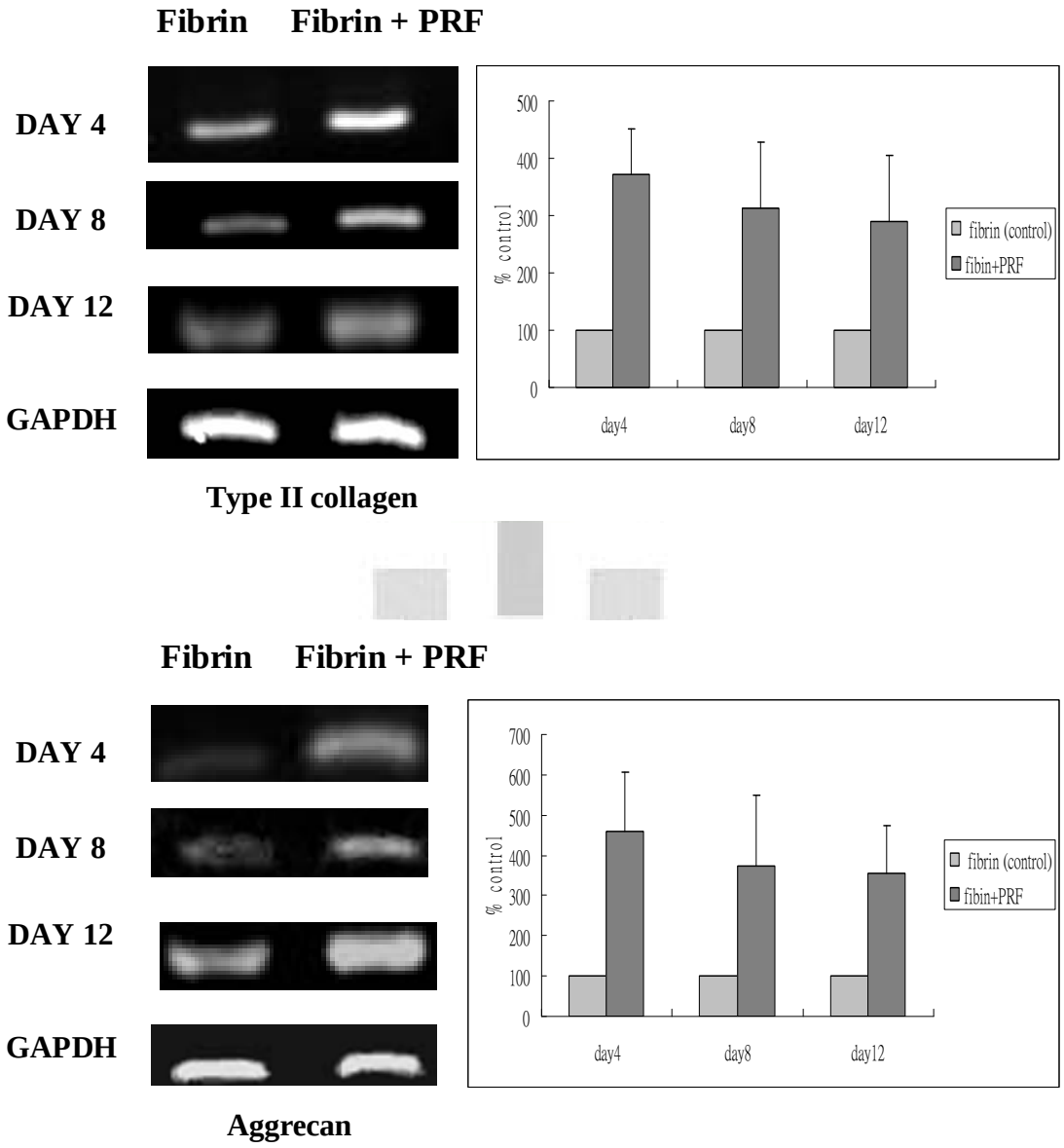

Fibrin
Fibrin + PRF
DAY 4
DAY 8
DAY 12
GAPDH
% control
500
400
300
200
100
0
day4
day8
day12
fibrin (control)
fibin+PRF
Type II collagen
Fibrin
Fibrin + PRF
DAY 4
DAY 8
DAY 12
GAPDH
% control
700
600
500
400
300
200
100
0
day4
day8
day12
fibrin (control)
fibin+PRF
Aggrecan

Fig. 10 : Type II collagen and aggrecan expression of SW-1353 cells and primary human chondrocytes seeded in fibrin and fibrin+PRF scaffold.

Mixed fibrinogen (10mg/ml, 250λ), thrombin (5U/ml, 250λ), and PRF exudates 50λ. SW-1353 cells and chondrocytes in fibrin gel and fibrin + PRF gel scaffold after 4,8,12 days.

(A) Increased type II collagen and aggrecan expression when SW-1353 cells in fibrin +PRF compared with fibrin scaffold by RT-PCR.

(B) Increased type II collagen and aggrecan expression when chondrocytes in fibrin +PRF compared with fibrin scaffold by RT-PCR.

Fig. 11A

Fibrin **Fibrin + PRF**

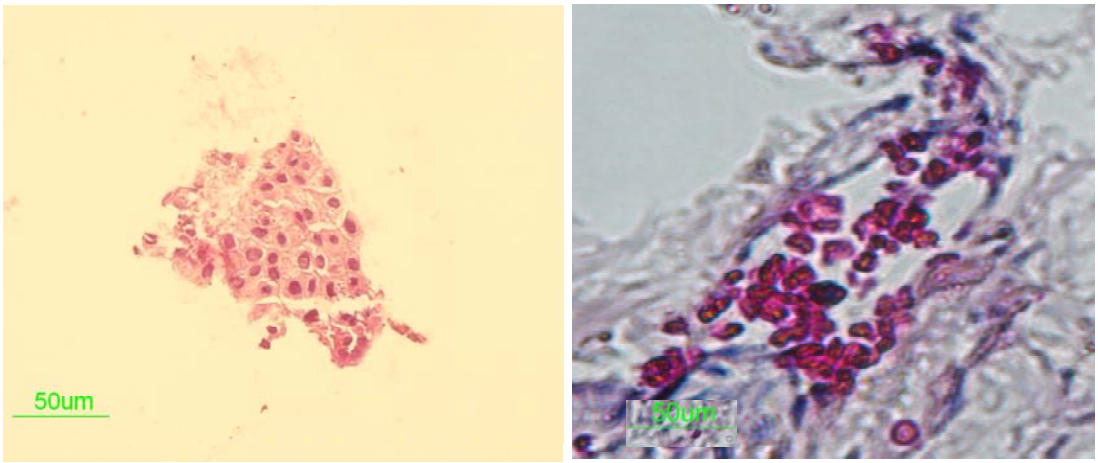
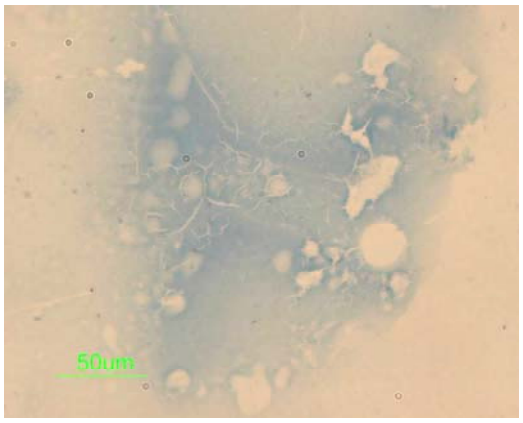
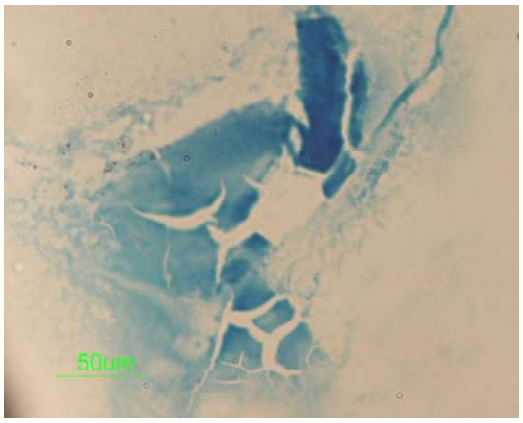


Fig. 11B

Fibrin **Fibrin + PRF**





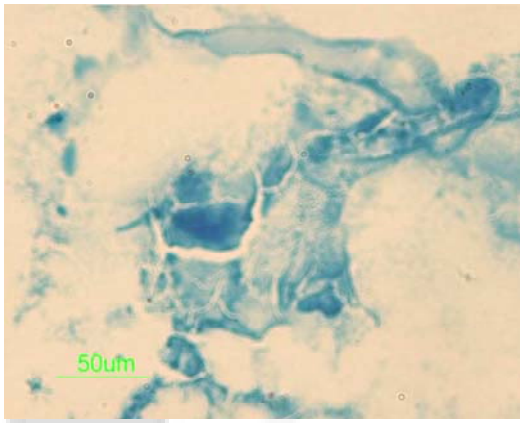
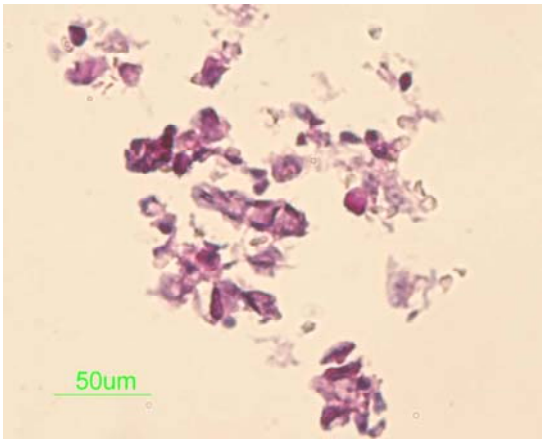


Fig. 11C

Fibrin **Fibrin + PRF**



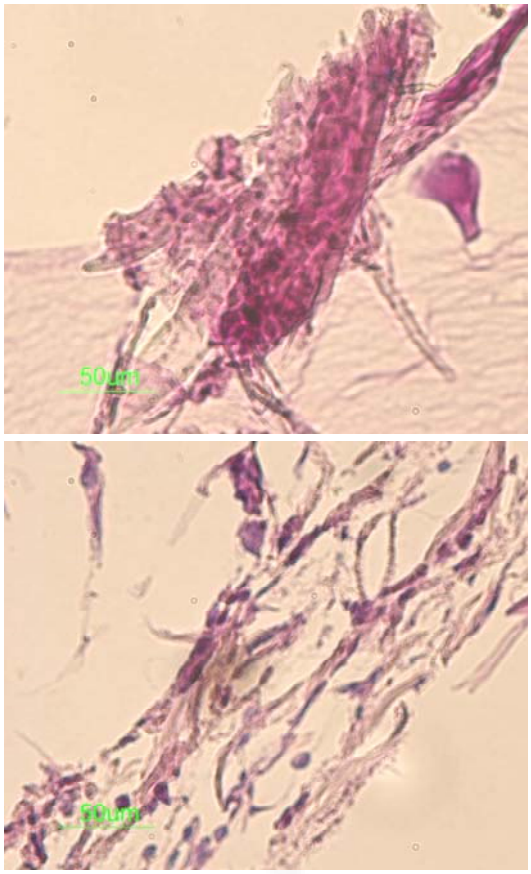


Fig.11 : Primary human chondrocytes cultivated in fibrin and fibrin + PRF 3D scaffold.

GAGs and proteoglycan accumulation of chondrocytes in fibrin + PRF scaffold were more than in fibrin scaffold after 8 days.

(A) The constructs were sectioned after 8 days and were subject to H&E staining. Magnification, 400x.

(B) The constructs were sectioned after 8 days and were subject to Alcian blue staining for GAGs accumulation.Magnification, 400x.

(C) The constructs were sectioned after 8 days and were subject to PAS staining for proteoglycans accumulation. Magnification, 400x.