

# EVALUATION OF THREE DIMENSIONAL CONSTRUCT ENGINEERED FROM POLY(LACTIC-CO-GLYCOLIC ACID)/FIBRIN HYBRID SCAFFOLD USING RABBIT BONE MARROW MESENCHYMAL STEM CELLS FOR OSTEOCHONDRAL DEFECT REPAIR

## Article history

Received

26 June 2015

Received in revised form

7 September 2015

Accepted

15 October 2015

\*Corresponding author

munirahshaban@

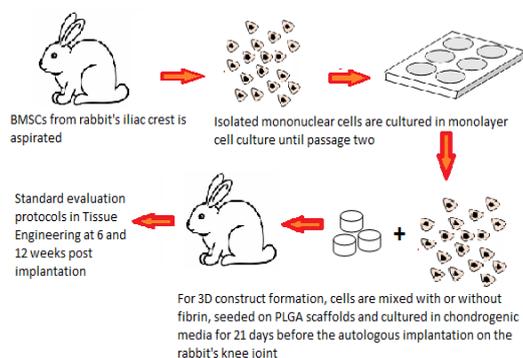
iium.edu.my

Rozlin Abdul Rahman<sup>a</sup>, Norhamiza Mohamad Sukri<sup>a</sup>, Noorhidayah Md Nazir<sup>a</sup>, Muhammad Aa'zamuddin Ahmad Radzi<sup>a</sup>, Ahmad Hafiz Zulkifly<sup>b</sup>, Aminudin Che Ahmad<sup>b</sup>, Suzanah Abdul Rahman<sup>a</sup>, Munirah Sha'ban<sup>a\*</sup>

<sup>a</sup>Department of Biomedical Science, Kulliyah of Allied Health Sciences, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang Darul Makmur, Malaysia

<sup>b</sup>Department of Orthopaedics, Traumatology and Rehabilitation, Kulliyah of Medicine, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, 25200 Kuantan, Pahang Darul Makmur, Malaysia

## Graphical abstract



## Abstract

Articular cartilage has poor repair capacity due to its avascular and aneural properties and has relatively few cells. This study investigated the ability of autologous implantation approach using three dimensional (3D) constructs engineered from bone marrow mesenchymal stem cells (BMSCs) seeded on poly(lactic-co-glycolic acid) (PLGA) with or without fibrin as cells carrier for the repair of osteochondral defect in rabbit model. The engineered 3D constructs – PLGA/Fibrin/BMSCs and PLGA/BMSCs – were cultured for 3 weeks *in vitro* and implanted autologously to the osteochondral defect created in the rabbit knee. The *in vivo* constructs were harvested and evaluated by means of gross observation, histology assessment, gene expression study, sulphated glycosaminoglycan (sGAG) production assay and biomechanical evaluation at 6 and 12 weeks post implantation. The results showed that the osteochondral defects treated with the PLGA/Fibrin/BMSCs constructs exhibited better repairment, more cartilaginous extracellular matrix, higher sGAG production, superior compressive strength and more intense expression of chondrogenic marker genes than the PLGA/BMSCs group. This study suggested that the PLGA/Fibrin/BMSCs has the potential to treat osteochondral defect and may be presented as a viable therapeutic option for those who would be in need from the life-extending benefits of tissue replacement or repair.

Keywords: Tissue engineering, poly(lactic-co-glycolic acid), fibrin, bone marrow mesenchymal stem cells, cartilage

© 2015 Penerbit UTM Press. All rights reserved

## 1.0 INTRODUCTION

Articular cartilage is a specialized connective tissue populated by highly specialized designed cells of mesenchymal origin. Being a unique structure however, articular cartilage is known to have a very limited capacity for self-repair because it lacks of blood vessel, lymphatic and nerve supply [1]. Therefore, treating damaged articular cartilage remains a challenge in the orthopaedic world.

The introduction of tissue engineering which aims to reconstruct functional tissue that resembles the native tissue has shown great potential in finding the answer to treat cartilage injury. It uses the principle of suitable cell sourcing, biomaterial scaffolds and signaling molecules. Recently, studies have shown that bone marrow mesenchymal stem cells (BMSCs) may serve as an attractive alternative in cartilage tissue engineering due to their differentiative, plasticity ability and easily accessible [2,3].

Previous study indicated that osteochondral defects filled with mesenchymal stem cells have shown good cartilaginous repair in rabbit models [4]. Since tissue engineering therapies are focuses on autologous treatment, the scaffold material become a fundamental component of an autologous transplant. A good scaffold should have these desired properties; bioactive, biodegradable, biocompatible, biomimetic and bioresponsive.

The use of PLGA and fibrin serve as a synergistic combo by combining both synthetic and natural biomaterial. It has been suggested that PLGA/Fibrin scaffold may serve as a potential cell delivery and as a structural platform for tissue engineered articular cartilage [5].

For this study, the potential of autologous implantation approach using 3D tissue construct engineered from rabbit BMSCs seeded with or without the presence of fibrin on PLGA scaffold for full-thickness osteochondral repair was evaluated by analyzing the implanted construct in order to provide an alternative for cartilage tissue engineering.

## 2.0 EXPERIMENTAL

### 2.1 Polymer Preparation

The PLGA (Sigma, Sigma-Aldrich, USA) co-polymer (mole ration 50:50, molecular weight 33 kg/mol, Resomer RG 503H) was used. Microporous 3D PLGA scaffold were prepared via solvent casting-salt leaching method using methylene chloride (Merck, Merck Schuchardt OHG, Germany) as previously described [6]. A silicone mould (7 mm in diameter and 3 mm in thickness) was used to make cylindrical shaped disk scaffolds.

### 2.2 Mesenchymal Stem Cell Isolation and Culture

BMSCs were harvested from 16-week-old New Zealand white rabbits. The marrow was aseptically

aspirated from the iliac crest and underwent gradient centrifugation method using Ficoll Paque™ (Ficoll-Paque™ Plus, GE Healthcare, Bio-Science AB, Sweden) to isolate the mononuclear cells in accordance with the manufacturer protocol. Cells were plated with the initial seeding of 10,000 cells/cm<sup>2</sup> in 6-well plates (Thermo Scientific, Nunclon Delta Surface, Denmark) supplemented with commercially available complete medium ChondroEnhance™ (ChondroEnhance™, TELA Technology, Malaysia). All cultures were maintained in a standard culture conditions of 37°C and 5% humidified CO<sub>2</sub> (Thermo Scientific, Barnstead Lab Line, USA). Passage two cells were used in all experiments.

### 2.3 Preparation of Fresh Plasma-Derived Fibrin

Fresh fibrin was obtained from the rabbit's plasma collected in buffered sodium citrate tubes (BD Vacutainer, BD, USA) by 2576g centrifugation for 6 minutes at 4°C (Mikro 220R, Hettich Zentrifugen, Germany).

### 2.4 Cell Seeding and Culture of Constructs In Vitro

100,000 cells per scaffold were incorporated and resuspended in (1) plasma-derived fibrin and (2) culture medium. PLGA scaffolds were soaked carefully in the BMSCs plasma-derived fibrin suspension (PFC group). For the control group, PLGA scaffolds were soaked in BMSC suspended in culture medium only (PC group). ChondroEnhance™ supplemented with 10<sup>-7</sup> M dexamethasone (Calbiochem, EMD Chemicals, USA) and 10% insulin transferrin selenium (Gibco, Life Technologies, USA) were used as the chondrogenic differentiation media [7]. Constructs were cultured for 21 days before the actual implantation.

### 2.5 Implantation of the Constructs into Rabbit Knee Joint Cartilage Defect

Thirty four male rabbits (2.5-3.0 kg) were used in this study. All animal procedures and operations were performed under standard guidelines approved by the IIUM Research Ethics Committee (IREC) (reference number: IIUM/305/20/4/10). A full-thickness defect (3mm in depth) was created at the medial and lateral femoral condyle by using a 3mm drill bit (Acculan GD 620D, B-Braun, Germany). The defect in the medial condyle was implanted with PFC construct and the lateral condyle was implanted with PC construct. At 6 and 12 weeks after implantation, the rabbits were euthanized.

### 2.6 Gross Morphology Assessment

The gross appearance was assessed and scored for the extend of defect repair, integration with the surrounding native cartilage and gross appearance using the International Cartilage Repair Society (ICRS) classification [8].

## 2.7 Histological and Immunohistochemistry Evaluation

The sections were stained with hematoxylin and eosin (H&E) for morphological evaluation, Alcian blue for glycosaminoglycan production, Safranin O for proteoglycan distribution and for type-II collagen immunostaining, the sections were incubated with a 1:1000 dilution of a monoclonal mouse anti-rabbit collagen type II (Calbiochem® EMD Biosciences Inc., La Jolla).

## 2.8 Two-Step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 100,000 to 1 million BMSCs isolated from the tissue engineered construct using TRIzol reagent (Invitrogen, Life Technologies, USA). Cell suspension in the TRIzol reagent was centrifuged at 12,000 rpm for 5 minutes at 4°C. The liquid phase formed was transferred into a new microcentrifuge tube (Purepak™, Molecular Bioproducts, Inc, USA). Chloroform (Sigma, Sigma-Aldrich, USA) was added and left for 10 minutes at room temperature. The mixture of liquid phase and chloroform was then centrifuged 12,000 rpm for 15 minutes at 4°C resulting into three distinct layer which consist of RNA, protein layer and DNA. The upper most layer containing RNA was transferred carefully into another microcentrifuge tube. Isopropanol (Sigma, Sigma-Aldrich, USA) was added and mixed thoroughly. Poly acryl carrier (Molecular Research Centre, Inc. Cincinnati, OH) was used to help precipitate the total RNA. The total RNA was washed with 75% ethanol and air-dried. Expression of cartilage specific markers namely the collagen type II, collagen type IX, aggrecan core protein, sox-9 and also cartilage dedifferentiation marker collagen type I were evaluated by using a two-step reverse transcription polymerase chain reaction (PCR) technique. Specific primers used are shown in Table 1.  $\beta$ -actin serves as an internal control. The reverse transcription protocol was carried according to the manufacturer's protocol using Superscript™ II Reverse Transcriptase (Invitrogen, Life Technologies, USA). For PCR, after the initial denaturation of the template at 94°C, polymerase chain reaction was performed for 38 cycles at 94°C for 30 seconds, at 52°C for 30 seconds, at 72°C for 30 seconds and final extension at 72°C for 5 minutes. The amplified PCR products were separated by 1.5% agarose gel (Vivantis, Vivantis Inc, USA) via electrophoresis (EC 300 XL, Thermo Scientific, USA) stained with ethidium bromide (EMSURE®, Merck KGaA, Germany) and visualized by UV transillumination using gel documentation system Alphamager® HP (Alpha Imager HP System, Alpha Innotech, USA).

## 2.9 Sulphated Glycosaminoglycan (sGAG) Production Assay

The sGAG content was determined by using K-Assay® sGAG assay kit (K-Assay®, USA) following the manufacturer's protocol. Total sGAG were

normalized by dried-weight of each sample (n=6) as a relative sGAG content (%).

## 2.10 Biomechanical Analysis

The biomechanical properties of the engineered construct (n=6) were analyzed on a biomechanical testing machine (Instron E300, Germany) at 12 weeks post implantation. A constant compressive strain rate of 1 mm/min was applied until failure and compressive stress was plotted to determine the compressive strength of the implanted constructs.

## 2.11 Statistical Analysis

All statistical analysis was done using SPSS version 15.0. The significant difference was set at  $p < 0.05$ . For the ICRS Classification, Kruskal-Wallis test was used to compare the three groups (PFC, PC and non-treated). For the sGAG assay and biomechanical analysis, data was expressed as mean  $\pm$  S.E.M using the Student's t-test to determine the statistical significance between groups.

## 3.0 RESULTS AND DISCUSSION

### 3.1 Macroscopic Observation using ICRS Classification

After 6 weeks post implantation, the defect margin was fairly visible in the PFC group but the defect was filled with shiny white regenerative tissue (Figure 1C). The PC group had minimal regenerative tissue and appeared centrally concaved (Figure 1B) while the non treated group remained empty (Figure 1A).

At 12 weeks after implantation, PFC exhibited superior regenerated appearance that integrated well with the surrounding tissue (Figure 1F). For PC, the defect was mostly covered with whitish tissue but the centre remained concaved (Figure 1E). The non treated group however had very minimal tissue reparation (Figure 1D). Studies have shown that when the repaired tissue and the native cartilage are well integrated, further cartilage damage could be avoided and this may provide a suitable biomechanical environment for the joint to withstand any forces [9,10].

For the ICRS scoring, at 6 weeks, PFC and PC had a median scores of 6 and 5 respectively and after 12 weeks the scores rose to 11 and 6 respectively, with PFC showed a significantly higher median score at both time points ( $p=0.043$ ;  $p=0.022$ ).

PFC and PC demonstrated a median score that corresponded to nearly normal and abnormal appearance while the non-treated group exhibited a severely abnormal appearance at 6 and 12 weeks respectively (Table 2).

Table 1 Primers used for RT-PCR

Genes	Primer Sequences	GenBank Accession No.	Product Size (bp)
Collagen type II	F:5'-CAACAACCAGATCGAGAGCA-3' R:5'-CCAGTAGTACCCGCTCTCC-3'	XM_002723439.2	112
Collagen type I	F:5'-GGTCCTCAGGGTCTTCTGG-3' R:5'-CACCAGGAGCACCGTTGACT-3'	NM_001195668.1	184
β-actin	F:5'-GCCATCCTGCGTCTGGACCTGGCT-3' R:5'-GTGATGACCTGGCCGTCAGGCAGC-3'	NM_001101683.1	227
Sox-9	F:5'-GGTGCTCAAGGGCTACGACT-3' R:5'-GGGTGGTCTTCTTGTGCTG-3'	XM_008271763.1	273
Collagen type IX	F:5'-ACTGGAGCAATCGGCTTCA-3' R:5'-CCCTCTCACCCCTGTGTC-3'	XM_008263089.1	158
Aggrecan core protein	F:5'-ATCAACAGAGACCTACGATGT-3' R:5'-GITAGGGGTAGAGGTAGACCGT-3'	XM_008251726.1	290

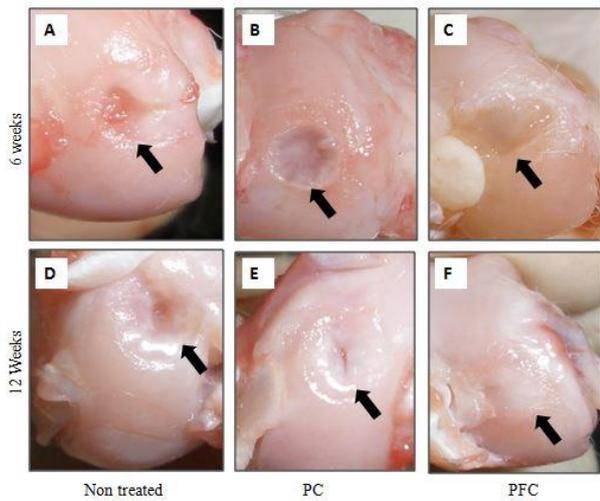


Figure 1 Gross morphology of the repaired defect at 6 weeks and 12 weeks post implantation. Arrow showed the location of defect area

Table 2 Gross morphology using ICRS Classification

Treatment	6 weeks post implantation	12 weeks post implantation
PFC	9*	11*
PC	5	6
Non treated	0	2

\*PFC group demonstrated significantly higher scores at both time points (p<0.05). The score for overall repair assessment using ICRS Classification: Grade I- normal; 12 points, Grade II- nearly normal; 11-8 points, Grade III- abnormal; 7-4 points, Grade IV- severely abnormal; 3-1 points

3.2 Histological and Immunohistochemistry Evaluation

At 6 week, PFC group showed relatively smooth surface and homogenous cell distribution (Figure 2A-D) with the evidence of cartilage extracellular matrix (ECM) (Figure 2A-E) and proteoglycan production (Figure 2A-F). PC group on the other hand had irregular surface with hypocellularity and the presence of cell clustering (Figure 2A-A). There was minimal cartilage ECM noted (Figure 2A-B) and Safranin O staining was negative (Figure 2A-C).

At 12 weeks, PFC group showed marked hypercellularity that blended with the surrounding

area (Figure 2B-D). Abundant cartilage ECM and proteoglycan were noted (Figure 2B-E and 2B-F). PC group however remained hypocellularized (Figure 2B-A) with weak expression of cartilage ECM and no Safranin O staining (Figure 2B-B and 2B-C).

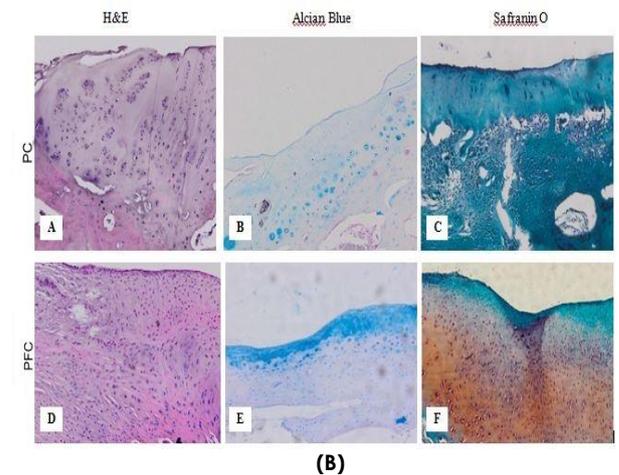
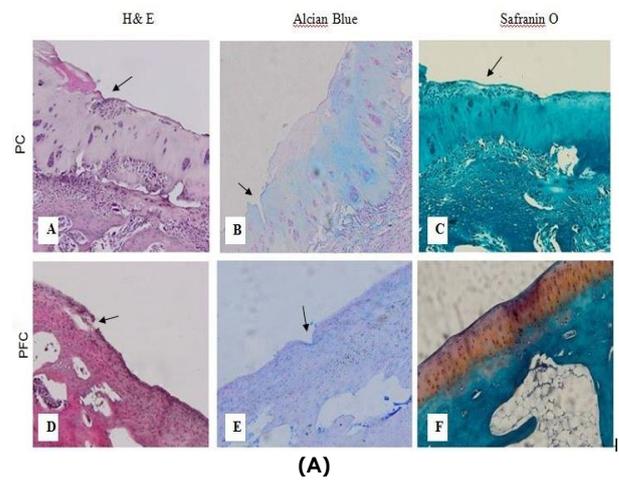
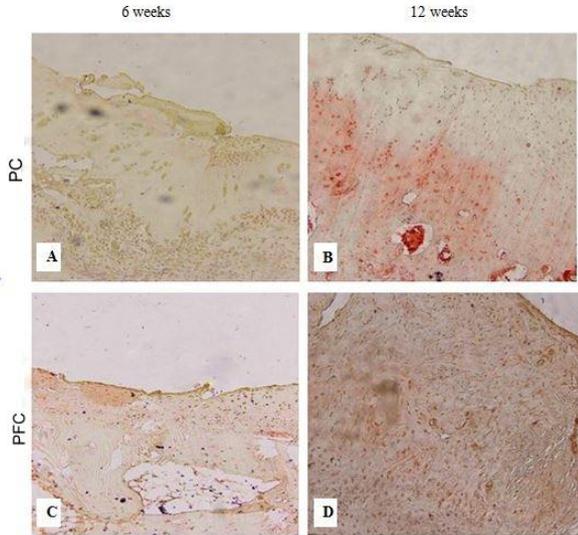


Figure 2 H&E, Alcian Blue and Safranin O staining of sections for PFC and PC group at (A) 6 weeks after implantation and (B) 12 weeks after implantation (magnification of 100X; scale bar: 100 μm).

The IHC staining for collagen type II revealed a stronger immunopositivity for PFC than PC at 6 and 12 weeks post implantation (Figure 3).

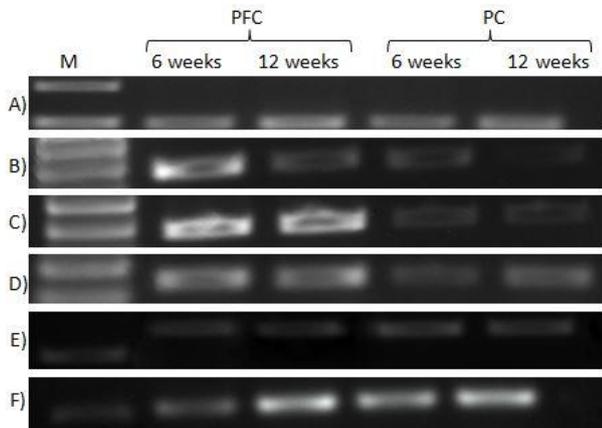
The marked superior histo-architecture in the PFC group signifies that the construct with the presence of fibrin, is able to hold the cells together [5], and that the combination of PLGA scaffolds and BMSCs potentiate the cartilage regeneration through cartilaginous matrix production and distribution which is consistent with previous studies [4, 11].



**Figure 3** Immunohistochemistry staining of collagen type II for PFC and PC at 6 weeks and 12 weeks post implantation (magnification of 100X; scale bar: 100 µm)

**3.3 Gene Expression Analysis**

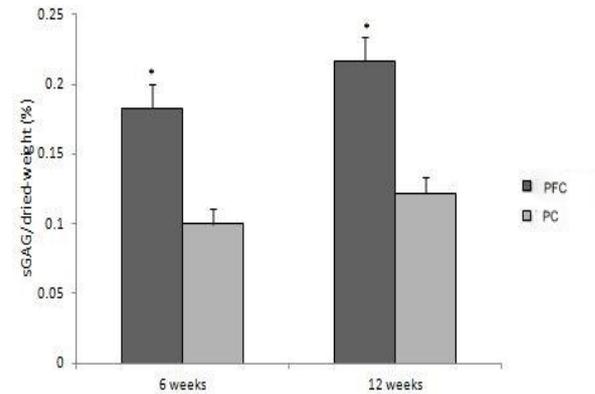
At both time points, the PFC and PC group managed to express the cartilage specific marker, mainly the collagen type II, sox9, collagen type IX and aggrecan mRNAs, with the PFC group demonstrated more intense and better expression compared to PC. Collagen type I, which is the cartilage dedifferentiation marker showed a relatively down-regulated pattern at week 12 in both groups. The co-existence of collagen type I together with collagen type II is seen in the early stage of chondrogenesis which later can be found very sparsely or completely absent in the hyaline cartilage [12]. The results are summarized in Figure 4.



**Figure 4** RT-PCR analysis of the mRNA expression of (A) Collagen type II, (B) Sox9, (C) Aggrecan core protein, (D) Collagen type IX, (E) Collagen type I and (F) β-actin (Note: **M**: 100bp DNA ladder)

**3.4 Sulphated Glycosaminoglycan (sGAG) Production**

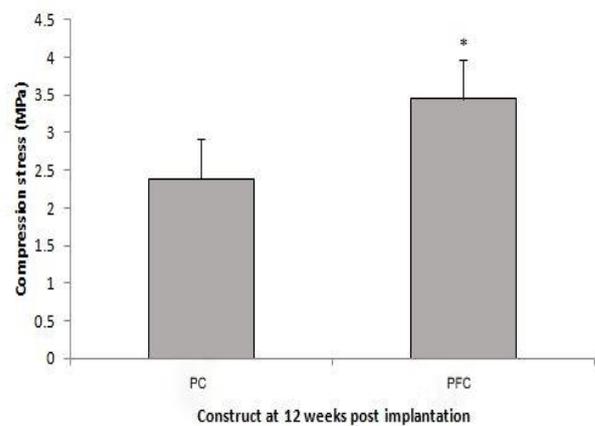
Figure 5 denotes that PFC group exhibited a significantly higher relative sGAG content amounting  $0.188 \pm 0.028$  compared to PC  $0.100 \pm 0.022$  at 6 weeks ( $p=0.028$ ). While at 12 weeks, PFC group managed to produce a 1.78 folds higher production of sGAG amounting  $0.217 \pm 0.038$ , which is significantly higher than PC;  $0.122 \pm 0.019$  ( $p=0.027$ ). This finding corresponded with our histology assessment discussed above.



**Figure 5** Production of sGAG at 6 weeks and 12 weeks after implantation. PFC produced significantly higher sGAG content at both time points ( $p<0.05$ )

**3.5 Biomechanical Testing**

At 12 weeks post implantation, PFC produced significantly higher mechanical strength reaching  $3.45 \pm 0.13$  MPa at 35% strains compared to PC that could only withstand until  $2.39 \pm 0.12$  MPa at 35% strains ( $p=0.028$ ). The result is summarized in Figure 6. The distinct differences in the biomechanical property between PFC and PC indicates that constructs with higher sGAG productive gives better load bearing function for articular cartilage [13].



**Figure 6** PFC group showed significantly higher mechanical strength than PC group ( $p<0.05$ ) when compression test was applied at 12 weeks post implantation

## 4.0 CONCLUSION

This study concluded that our PLGA/Fibrin hybrid scaffolds when seeded with BMSCs have the potential to regenerate articular cartilage in an osteochondral defect by enhancing cell distributions and integration as well as higher cartilage ECM production whilst providing a good biomechanical property in a rabbit model.

## Acknowledgement

The authors would like to thank Kulliyah of Allied Health Sciences (KAHS), and Kulliyah of Medicine (KOM), International Islamic University Malaysia (IIUM) and the Exploratory Research Grant Scheme (ERGS) from the Ministry of Higher Education (MOHE) Malaysia (Grant No. ERGS 11-005-0005)

## References

- [1] Pearle, A. D., Warren, R. F., Rodeo, S. A. 2005. Basic Science of Articular Cartilage and Osteoarthritis. *Clinics in Sports Medicine*. 1-12.
- [2] Vinatier, C., Bouffi, C., Merceron, C., Gordeladze, J., Brondello, J-M, Jorgensen, C., et al. 2009. Cartilage Tissue Engineering: Towards a Biomaterial-Assisted Mesenchymal Stem Cell Therapy. *Curr Stem Cell Res Ther*. 4(4): 318-29.
- [3] Seo, S., Na, K. 2011. Mesenchymal Stem Cell-Based Tissue Engineering for Chondrogenesis. *J Biomed Biotechnol*. 2011: 806891.
- [4] Uematsu, K., Hattori, K., Ishimoto, Y., Yamauchi, J., Habata, T., Takakura, Y., et al. 2005. Cartilage regeneration Using Mesenchymal Stem Cells and a Three Dimensional Poly-Lactic-Glycolic Acid (PLGA) scaffold. *Biomaterials*. 26(20): 4273-9.
- [5] Abdul Rahman, R., Mohamad Sukri, N., Md Nazir, N., Ahmad Radzi, M. A., Zulkify, A. H., Che Ahmad, A. et al. The Potential of 3-dimensional Construct Engineered from Poly(lactic-co-glycolic acid)/fibrin Hybrid Scaffold Seeded with Bone Marrow Mesenchymal Stem Cells for in Vitro Cartilage Tissue Engineering. *Tissue Cell*. 2015. doi: 10.1016/j.tice.2015.06.001.
- [6] Munirah, S., Kim, S. H., Ruszymah, B. H. I., Khang, G. 2008. The Use of Fibrin and poly(lactic-co-glycolic acid) Hybrid Scaffold for Articular Cartilage Tissue Engineering: An in Vivo Analysis. *Eur Cells Mater*. 15: 41-52.
- [7] Solchaga, L. A., Penick, K. J., Welter, J. F. 2011. Chondrogenic Differentiation of Bone Marrow-Derived Mesenchymal Stem Cells: Tips and Tricks. *Methods Mol Biol* [Internet]. 698: 253-78.
- [8] Van den Borne, M. P.J., Raijmakers, N. J. H., Vanlauwe, J., Victor, J., de Jong, S. N., Bellemans, J. et al. 2007. International Cartilage Repair Society (ICRS) and Oswestry Macroscopic Cartilage Evaluation Scores Validated for Use in Autologous Chondrocyte Implantation (ACI) and Microfracture. *Osteoarthritis Cartil*. 15(12): 1397-402.
- [9] Madry, H., Kaul, G., Cucchiari, M., Stein, U., Zurakowski, D., Remberger, K., et al. 2005. Enhanced Repair of Articular Cartilage Defects in Vivo by Transplanted Chondrocytes Overexpressing Insulin-Like Growth Factor I (IGF-I). *Gene Ther*. 12(15): 1171-9.
- [10] Chen, J., Wang, C., Lü, S., Wu, J., Guo, X., Duan, C., et al. 2005. In vivo Chondrogenesis of Adult Bone-Marrow-Derived Autologous Mesenchymal Stem Cells. *Cell Tissue Res*. 319(3): 429-38.
- [11] Qi, Y., Du, Y., Li, W., Dai, X., Zhao, T., Yan, W. 2014. Cartilage Repair Using Mesenchymal Stem Cell (MSC) Sheet and Mscs-Loaded Bilayer PLGA Scaffold in a Rabbit Model. *Knee Surgery, Sport Traumatol Arthrosc*. 22(6): 1424-33.
- [12] Morrison, E. H., Ferguson, M. W. J., Bayliss, M. T., Archer CW. 1996. The Development of Articular Cartilage: I. The Spatial and Temporal Patterns of Collagen Types. *J Anat*. 189: 9-22.
- [13] Connelly, J. T., Wilson, C. G., Levenston, M. E. 2008. Characterization of Proteoglycan Production and Processing by Chondrocytes and Bmscs in Tissue Engineered Constructs. *Osteoarthritis Cartil*. 16(9): 1092-100.