

**Open Access** 

# Modulation of Chondrocyte Phenotype by Bioreactor Assisted Static Compression in a 3D Polymeric Scaffold with Potential Implications to Functional Cartilage Tissue Engineering

#### Remya NS and Prabha D Nair\*

Scientist G, Division of Tissue Engineering & Regeneration Technologies, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Trivandrum-695012, India

\*Corresponding author: Prabha D Nair, Scientist G, Division of TissueEngineering & Regeneration Technologies, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences & Technology, Trivandrum-695012, India, Tel: 91- 471 – 2520242; E-mail: pdnair49@gmail.com

Received date: December 03, 2014; Accepted date: January 30, 2015; Published date: February 03, 2015

**Copyright:** © 2015 Nair PD et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

#### Abstract

Functional cartilage tissue engineering aims at augmenting the regeneration process of chondrocyte seeded three dimensional scaffolds by application of external biomechanical stimuli. The effect of static compressive stimuli in modulating the phenotype of chondrocytes cultured in porous scaffolds using a bioreactor is being investigated in the present study. Chondrocytes were seeded in porous Poly (vinyl) alcohol-Poly capro lactone scaffold and was subjected to static unconfined compressive strain of 10% for 1h everyday for a period of 7days using a bioreactor. After culture period, chondrogenic phenotype of seeded cells was assessed by live dead assay, biochemical histological and real time PCR analysis. Bioreactor seems to be a promising tool in delivering the desired biomechanical stimuli to the cell seeded constructs. However, application of biomechanical stimuli in the form of static compression doesn't seem beneficial as it modulates chondrogenic phenotype by reverting to a fibroblastic morphology with the secretion of collagen type 1 extra cellular matrix molecules

**Keywords:** Bioreactor; Cartilage tissue engineering; Chondrocytes; Static compression; Three dimensional scaffolds; Chondrocyte phenotype; Biomechanical stimuli

#### Introduction

The treatment of articular cartilage defects still remains a challenge owing to the intrinsic nature of articular cartilage as well as the short comings of currently available treatment modalities [1]. Tissue engineering approach exploits the possibility of using in vitro cultured chondrocytes in suitable three dimensional matrix [2].When these in vitro cartilage constructs are implanted into a cartilage defect, they may induce the successful regeneration of functional cartilage. Various natural and synthetic polymers have been extensively studied for their suitability in serving as a three dimensional matrix to support the growth of chondrocytes [3]. In addition to biochemical cues, cell- cell as well as cell- matrix interactions plays a very important role in generating functional tissue in vitro [4]. However, these in vitro constructs seems to be inferior when compared to the structural, functional and biomechanical properties of natural articular cartilage tissue.

Natural articular cartilage is exposed to different types of biomechanical stimuli in vivo under normal physiological conditions. Often their magnitude differs as in routine activities such as walking, running or climbing stairs [5]. Static physiological stresses applied to knee joints in standing position even for 5-30 minutes can increase the compressive strain up to 40% in knee joints [6]. The stress and /or strain experienced by the cells and the interstitial fluid flow resulting from mechanical loading can have different effects on chondrocyte activity. It can up or down regulate synthesis of different extra cellular matrix macromolecules, can also affect the production of agents

involved in matrix breakdown which is accompanied by deformational changes in chondrocyte phenotype.

The concept of functional tissue engineering introduces the benefit of biomechanical stimuli in the in vitro engineering of cartilage in improving the quality of constructs. Accordingly the mechanical environment of chondrocytes can best be reproduced *in vitro* by direct compression; and direct static compression simulates the normal physiological condition as that of standing. Hence this *in vitro* study examined the effect of application of a static compressive load to chondrocytes after the cells had been seeded onto a porous three dimensional scaffold.

### Methodology

#### Isolation and culture of rabbit chondrocytes

Chondrocytes were isolated from rabbit articular cartilage by following an established protocol [7]. Briefly, the articular joints were collected aseptically and transferred in PBS containing antibiotics. The cartilage pieces, after exposing the joints, were scrapped, washed in PBS and digested in chondrocyte digestion media (DMEM, Collagenase type II 0.2% (w/v), containing penicillin (100 units/mL), streptomycin (100 mg/ mL) and fungizone (1 mg/mL)) for 16 h at 200 rpm in an orbital shaker at 37°C. After digestion the collagenase action was inhibited using resuspension media (DMEM, 10% FBS). The suspension was centrifuged at 1200 rpm for 15 min to get a pellet and resuspended in chondrogenic culture media (DMEM supplemented with 10% FBS, penicillin (100 U/mL), streptomycin (100 mg/mL), sodium pyruvate (1 mM/mL), glutamine (1.4 mM/mL), NEAA (0.1 mM/mL), ascorbic acid (50 mg/L), proline (40 mg/L) and incubated at 37°C, 5% CO2 until confluent. Medium was changed every 2-3 days following which the confluent monolayer was trypsinized using 0.25%

#### Page 2 of 5

Trypsin- EDTA solution. Cells obtained at the third passage were used for further studies.

# Fabrication and characterization of PVA-PCL scaffold

Poly (vinyl alcohol)-Poly (caprolactone) semi-interpenetrating polymer network scaffolds for the study were prepared as per the established protocol[7]. Briefly, the scaffold was fabricated by high speed mixing of polyvinyl alcohol (aqueous solution) and poly caprolactone (in chloroform) at equal concentrations using a mechanical stirrer to form homogeneous foam. The foam was immediately transferred to moulds, frozen, and lyophilized at -80°C for 24 h .The porous scaffolds were cross linked with 1% gluteraldehyde solution to form the semi- interpenetrating network, washed to remove traces of gluteraldehyde and lyophilized before use. The lyophilized scaffolds were cut into discs of desired size, sterilized by ethylene oxide and stored till use. The scaffolds were physicochemically characterized to demonstrate its porous structure, swelling ability, chemical composition and biodegradation profile using various techniques as reported accordingly [7].

# **Experimental setup**

PVA-PCL scaffolds were seeded with approximately 1x10<sup>6</sup> chondrocyte per scaffold (6mm diameter and 2mm thickness) and cultured at 37°C in a CO2 incubator. After 3 days of static culture half of the cell seeded constructs were transferred to a bioreactor (Cartigen, Tissue Growth Technologies, USA) for mechanical stimulation )as dictated by the digital software controller( Growth Works<sup>\*</sup>). The cell seeded constructs in chondrogenic medium were given static unconfined compressive strain of 10% for 1hour for a period of 7days. Medium was changed every 2 days.

### Evaluation of tissue engineered constructs

**Cell viability- Live dead assay:** The viability and membrane integrity of chondrocytes under loading, were determined using LIVE/DEAD<sup>\*</sup> viability/Cytotoxicity kit The constructs were incubated in DMEM containing 4mM Calcein-AM and 2 Mm Ethidium homodimer-1 for 30 min. Live cells are permeable to the non-fluorescent Calcein AM which is converted into fluorescent dye Calcein by cytoplasmic esterases present in living cells and appear as green. On the other hand, Ethidium homodimer-1 enters the cell lacking membrane integrity fluoresces when bound to nucleic acid and is shown red. The imaging was done using Fluorescent microscope.

# **Biochemical analysis**

Quantitative estimation of glycosaminoglycan (GAG), total collagen and DNA were done on papain digested constructs according to accepted protocol. Briefly, the constructs were digested using papain (pH 6.5) at 65 0 C for 4hours for complete digestion and the supernatant was used for the assay. Total collagen was estimated using Sircoll assay kit based on manufacturer's instructions. Total GAGs were quantified by Dimethyl methelyene blue (DMMB) assay and DNA concentration was determined by picogreen assay according to the kit protocol.

# Histological analysis

The retrieved samples were processed for staining of the sections using standard histopathology protocol. Briefly, the retrieved

constructs were fixed in formalin free fixative for 24 hours and dehydrated by passing through an increasing series of ethanol (50%, 70%, 90% and 100%) and then in xylene. Processed sample was oriented and embedded in paraffin wax for 60 minutes. Sections of 4 micron thickness were cut using a microtome The sections were stretched out on a water bath at 45°C, mounted on microscope slides and kept to dry in an air oven set at 37°C overnight. The sections were deparaffinised in xylene for 5 minutes and rehydrated by passing through a series of alcohol (100%, 90%, 80%, 70%) and finally in distilled water. Safranin-O staining was employed for visualizing glycosaminoglycans. Extra cellular matrix was also analyzed by staining with Toluidine blue. Immuno staining for collagen type 11 was done using specific antibody against collagen type 11.

# **Real Time PCR analysis**

Total RNA was isolated using Trizol reagent according to the manufacturer's instructions. The yield and purity were determined using UV Spectrophotometer. The first-strand cDNA was then synthesized from total RNA, by a reverse transcription (RT) reaction using the Thermal CyclerFor quantitative determination, the real-time PCR was performed by syber green assay at an annealing temperature of 58°C (depending upon the primers) over 35 cycles. Expression of collagen Type II, aggre can and collagen 1 were assessed for chondrogenesis. Glyceraldehyde phosphate dehydrogenase was used as the house keeping gene. For each gene, the quality and specificity were assessed by examining polymerase chain reaction (PCR) melt curves after real-time PCR. The relative level of expression was calculated with the  $\Delta\Delta$ Ct method [8].

### Statistics

Data were analyzed using one-way ANOVA and post-hoc pair wise t-tests. Differences were considered significant at or below the p<0:05 level.

# **Results and Discussion**

### **PVA-PCL scaffolds**

The PVA-PCL scaffold was fabricated by high speed phase mixing of PVA in water and PCL in chloroform followed by freeze drying and cross linking of PVA groups by gluteraldehyde. The scaffold is found to be mimicking the properties of natural articular cartilage, wherein PCL imparting mechanical strength and PVA contributing to the water holding capacity. The morphology of the porous PVA-PCL scaffolds was observed by SEM (Figure 1a). The pore sizes were within the range of 30-300µm and were interconnecting. The scaffold showed a percentage porosity of 70.7%, average wall thickness of 0.0591, and average pore diameter of 0.09 mm and anisotropy of 1.117 when evaluated by Micro CT (Figure 1b). To engineer a load bearing tissue like cartilage, the key issue is the magnitude of load bearing required from the construct. PVA-PCL as reported in the earlier studies [7] withstands a compressive stress of 0.303 MPa, and has a Youngs modulus of 1.78 MPa and hence possesses sufficient mechanical strength to withstand the load experienced in the joints during normal daily activities. The loading regime selected to evaluate the effect of static compression was 10% compressive strain for 1h for a period of 14 days. The preliminary tests on PVA-PCL scaffold confirmed that the scaffolds can sustain the selected compressive strain in this study (data not shown).

#### Page 3 of 5



**Figure 1:** a) Morphology of the porous PVA-PCL scaffolds as observed by SEM, 1b) Micro CT images of PVA-PCL scaffolds

# Evaluation of chondrocyte phenotype after static compression

The viability of cell seeded constructs was evaluated after culture by live dead assay and imaged using confocal microscopy (Figure 2). The cells were viable in both the conditions. However with mechanical loading the amount of dead cells were increased as evidenced by the presence of red cells in constructs after live dead assay, The findings were confirmed by quantitating the DNA content of the constructs. The DNA content was significantly low in loaded constructs compared to the free swelling culture conditions (p<0.01) (Figure 3). Mechanically loaded constructs had a DNA content of 19.6  $\pm$  0.03 µg while the amount of DNA in free swelling constructs was  $25.1 \pm 1.05$ µg. Moreover the cells were seen clustered and more towards the interior rather than on the surface as in free swelling conditions. A slight change in morphology was evident from the confocal images. The chondrocyte assumed a fibroblastic morphology in loaded constructs in contrast to the spherical morphology in free swelling culture constructs. Depth coding profile of free swelling as well as mechanical loaded constructs showed that viable cells were present even up to a depth of 120µm in mechanically loaded constructs whereas the cells penetrated to a depth of only 30µm in case of free swelling control (Figure 4).

The amount of GAG deposited in the chondrocyte/scaffold constructs after 2-week compression is shown in Figure 5a. It was observed that after static compression, GAG content decreased compared with the free-swelling samples (p<0.01).GAG content of free swelling constructs were 27.9  $\pm$  2.3 µg/10 µg of DNA while that of mechanically loaded constructs were 12.3  $\pm$  0.6 µg/10 µg of DNA. On the other hand total collagen, yet another biochemical marker of mechanically loaded constructs were higher than that of free swelling constructs. Total collagen content of mechanically loaded constructs

was  $1.2 \pm 0.007 \ \mu\text{g}/10 \ \mu\text{g}$  of DNA and free swelling was  $0.4 \pm 0.005 \ \mu\text{g}/10 \ \mu\text{g}$  of DNA respectively (Figure 5b).



Free swelling constructs

Mechanically loaded constructs

**Figure 2:** Live dead assay image of the cell seeded constructs under free swelling and mechanically loaded conditions (green cells represents live cells and red cells represents dead cells)







Free swelling constructs

Mechanically loaded constructs

**Figure 4:** Confocal imaging of cell seeded constructs: Depth coding profile -Color corresponds to the depth from the polymer surface, with blue being closest to the surface and red being at 120  $\mu$ m from the surface.

The expression of collagen type II, aggrecan and collagen type I were evaluated by RT PCR analysis to predict the modulation of functional phenotype of chondrocytes by static compression. The data is represented in Figure 6. A significant decrease in collagen type II expression was seen with static compression, when compared to free swelling constructs. The relative expression of collagen type II with respect to housekeeping gene GAPDH was 7.2  $\pm$  1.5 and 4.5  $\pm$  0.2 for

Page 4 of 5

the free swelling construct and mechanically loaded constructs respectively.



**Figure 5:** Biochemical analysis of the constructs a) Total glycosaminoglycans by DMMB assay b) Total collagen by Sirius red assay

Aggrecan, yet another chondrocyte extracellular matrix molecule also showed decreased expression in mechanically loaded cells when compared to the free swelling ones. The free swelling constructs showed an expression of 3.26±0.4 while loaded cells showed an expression of only 0.68±0.13. Interestingly, collagen type I, a marker for dedifferentiation or fibro cartilagenous formation was significantly increased in mechanically loaded cells (10.4±0.2) while it was minimal in free swelling constructs (0.5±0.005).



Figure 6: Relative gene expression of chondrocyte specific matrix molecules

Figure 7 shows the staining of mechanically loaded as well as free swelling controls. From the images it is clear that the cells are penetrated towards the interior of the scaffolds in mechanical loaded groups with the elaboration of matrix, while in the free swelling controls, the cells were seen mainly towards the boundary of the scaffold Also immunostaining of collagen type 11 showed the chondrocyte specific marker collagen type 11 in both the cell seeded constructs (Figure 8).

The main function of the cartilage matrix is to transmit load. Mechanical load is important in regulating the metabolic activity of chondrocytes, permitting them to adapt to their physical environment. This is essential both to maintain normal matrix properties and to preserve the main load-bearing function of cartilage. However, in the functional cartilage tissue engineering concept which makes use of external biomechanical stimuli in the form of compression to enhance the properties of neo cartilage formed *in vitro*, a clear understanding on the extent of compressive stimuli used seems to be beneficial in predicting the outcome.



**Figure 7:** Toluidine blue staining of cell seeded constructs demonstrating the GAG rich extracellular matrix a) Mechanically loaded constructs b) free swelling constructs



**Figure 8:** Immunostaining for collagen type 11 a) Mechanically loaded constructs b) free swelling constructs

As observed, the chondrocytes are very sensitive to mechanical loading. The differences in the magnitude or frequency of load can have different effects on chondrocyte activity and can up- or down regulate synthesis of different matrix macromolecules, and can also affect the production of agents involved in matrix breakdown. This is accompanied by some deformational changes in chondrocyte shape. Chondrocytes assumed a typical fibroblastic shape in contrast to the normal spherical shape under static compression. The typical extra cellular markers of hyaline cartilage, collagen type II and aggrecan was seen decreased while collagen type I, a marker for dedifferentiation or fibro cartilagenous formation was seen increased. This accounts for the increase in the total collagen content of the constructs. The increase in the collagen content increased the stiffness of the cartilage, which induced increased cell strain that may account for the change in the morphology of chondrocytes. GAGs or glycosaminoglycans were not able to aggregate under static compression and hence could not be retained inside the scaffold, thereby decreasing the total GAG content of the constructs

Chondrocytes will respond to excessive mechanical signals by disrupting the composition and structure of the extracellular matrix which reduces the biomechanical integrity of cartilage. The changes in biosynthetic activity with changes in cell density and axial tissue strain

Page 5 of 5

as well as the associated fluid loss from the matrix are important in regulating chondrocyte metabolism during static compression. Hence it could be concluded that mechanical loading in the form of static compression is not beneficial for the neo cartilage formation by the functional cartilage tissue engineering strategy.

# Conclusion

It has been widely recognized that mechanical loading plays a very important role in maintaining healthy joints and normal tissue remodelling. Furthermore, various in vitro studies also confirm the finding that moderate mechanical loading is necessary to maintain healthy cartilage. But however, if joints are insufficiently loaded, chondrocyte metabolism shifts in favour of catabolism and on the other hand, traumatic or excessive joint loading leads to cartilage degeneration and osteoarthritis. This study therefore gives evidence of chondrocytes responding to static compression by disrupting the composition and structure of the extracellular matrix thereby reducing the biomechanical integrity of cartilage

# Acknowledgments

The authors acknowledge the Director and Head, BMT Wing, Sree Chithra Tirunal Institute for Medical Sciences and Technology, India for the facilities provided, Dept. of Biotechnology, COE Programme in Tissue Engineering for financial assistance, and Remya NS

acknowledges the Indian Council for Medical Research for a SRF fellowship.

# References

- Chen FH and Tuan RS (2008) Adult Stem Cells for Cartilage Tissue Engineering and Regeneration. Current Rheumatology Reviews 4: 161-170.
- 2. Handschel JR. (2006)Depprich, Prospects of micromass culture technology in tissue engineering.Head & Face Medicine 3: 4.
- 3. Nair LS, Laurencin CT(2007)Biodegradable polymers as biomaterials. Progress in Polymer Science 32: 762-798.
- 4. Farshid G (2000) The deformation behavior and viscoelastic properties of chondrocytes in articular cartilage Biorheology 37: 27-44.
- Athanasiou KA, Darling EM(2009)Articular Cartilage Tissue Engineering Synthesis Lectures on Tissue Engineering 1: 1-182.
- Bader DL, Salter DM, Chowdhury TT (2011)Biomechanical Influence of Cartilage Homeostasis in Health and Disease. Arthritis Article ID 979032.
- Mohan N, Nair PD (2008)Polyvinyl alcohol-poly(caprolactone) Semi IPN scaffold with implication for cartilage tissue engineering. Journal of Biomedical Materials Research Part B: Applied Biomaterials 84B: 584-594.
- Dussault AA, M Pouliot(2006) Rapid and simple comparison of messengerrna levels using real-time PCR. Biological Procedures Online 8: 1-10.