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Research Article

Dynamic Compression Loading Aids in Chondrogenesis of Human Wharton's Jelly Mesenchymal Stem Cells Seeded on Chitosan-Agarose

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Abstract

Tissue engineering of articular cartilage is focused on generating the microenvironment, so as to restore its complex biomechanical and biochemical properties. We developed a novel Chitosan-Agarose (CHAG) scaffold resembling the properties of native cartilage extracellular matrix that aids *in-vitro* cartilage formation. Human Wharton's Jelly Mesenchymal Stem Cells (HWJ-MSCs) were seeded on CHAG scaffolds, cultured in Chondrogenic medium with supplementation of growth factors (BMP-2, TGF-β3). Dynamic compression loading was applied to mimic the microenvironment of native cartilage. The HWJ-MSCs seeded CHAG scaffolds have good cell attachment, infiltration and chondrogenesis with neo extracellular matrix synthesis. CHAG scaffolds seeded with HWJ-MSCs cultured in Chondrogenic media supplemented with both BMP-2 and TGF-β3 and simultaneous dynamic compression loading produced 15.26±2.48 µg GAG/µg DNA. Our results highlighted the potential strategy of using the combination of dynamic compression loading and CHAG scaffold in creating a functional, tissue engineered construct for articular cartilage.

Introduction

Articular cartilage, the smooth glistening white tissue covering the ends of articulating bones distributes the stress and reduces friction between bones. It has limited capacity to heal upon damages due to injuries or diseases [1-4]. Cartilage tissue engineering uses the combination of cells, scaffolds, growth factors and biomechanical factors to form neo-tissues so as to serve as alternative treatment modalities of the arthroscopic procedures like micro-fracture, osteochondral graft transplantation and autologous chondrocytes implantation [5-7]. The scaffold for tissue engineering should mimic the extracellular matrix of [8,9] and get replaced with the cartilage-like ECM upon degradation of scaffolds. Various natural and synthetic biomaterials have been reported so far as scaffolding material for cartilage tissue engineering *in-vitro* [10-12]. We have earlier reported on our development of a scaffold based on Chitosan-Agarose (CHAG) which has the required properties of resilience and modulus mimicking the properties of required for cartilage tissue engineering (under review JBMR-A).

Growth factors like TGF-β3 and Bone Morphogenetic Protein-2 (BMP-2) are two important growth factor of the TGF super family which plays an active role in cartilage formation and differentiation of Mesenchymal Stem Cells (MSCs) to Chondrogenic lineages [13-15]. The combination of BMP-2, and TGF-β3 have been used to differentiate rat bone marrow MSCs to chondrocytes [11,16,17]. Many reports have shown that the micro-environment alter the biomechanical properties and metabolic activity of the articular cartilage, increasing the Proteoglycans synthesis in chondrocytes-seeded agarose which when subjected to dynamic loading [18-21]. Here in we first report that Wharton's Jelly Mesenchymal Stem Cells (HWJ-MSC) seeded on to CHAG scaffolds, upon exposure to a microenvironment of dynamic compression loading and growth factor (BMP-2, TGFβ3) supplementation to the Chondrogenic medium promotes better differentiation of HWJ-MSCs to Chondrogenic lineage, creating cartilage like tissue formation.

Materials and Methods

Materials

Chitosan, DAPI, TFGβ3, BMP-2 were purchased from Sigma Aldrich (USA), Agarose (type I) was obtained from SRL (India), acetic acid were from Merck, (Germany), and DMEM, DMEM: F12 medium, and fetal bovine serum (FBS), 0.25% Trypsin EDTA, antibiotic and antimicotic -100X (ABAM) were from Gibco (USA). Collagen II specific monoclonal antibodies were obtained from abcam (USA), FITC conjugated secondary antibodies and DAPI were obtained from invitrogen

(USA), Wharton's Jelly Mesenchymal Stem Cells (HWJ-MSC) was procured from Himedia Labs, India, and mouse connective fibroblast cells (L929) from ATCC USA. All other solvents and reagents were of analytical grade.

Fabrication of Chitosan-Agarose (CHAG) scaffolds and characterization

Chitosan-Agarose scaffolds (CHAG) were prepared as described in (under review JBMR-A). Briefly, 4% chitosan (w/v) in 0.1M acetic acid, and 2% agarose (w/v) in distilled water were prepared, filtered with filter cloth (100µm mesh). Equal volume of the above solutions were added and mixed together under agitation (500 rpm) for 1 hour, poured in polypropylene dishes, freeze dried, treated with 0.1M NaOH, washed extensively in distilled water and again freeze dried to get CHAG scaffolds. The scaffolds were cut to 5mm diameter, 5mm thickness and used for the study unless it is described. Compression modulus, FT-IR, thermal analysis, Scanning electron microscopy, water uptake ability, *in-vitro* degradation, µct and cytotoxicity analysis have been studied and described in (under review JBMR-A).

Culture of Human Wharton Jelly-Mesenchymal Stem Cells (HWJ-MSCs)

Human Wharton Jelly-mesenchymal stem cells (HWJ-MSC) were purchased from Himedia labs, India and expanded in T500 flasks seeded at 5000 cells per cm^2 with stem cells expansion medium (Himedia, India), incubated at 37°C and 5% CO_2. The medium was changed every third day. At 80% confluency the cells were lifted using 0.025% trypsin-EDTA (Invitrogen, USA) and sub-cultured in expansion medium. The cells at passage 4 were used for all the chondrogenesis studies.

Cell Adhesion

CHAG scaffolds were seeded with 1×10^5 L929 cells or HWJ-MSC and cultured for 3 day. After the culture period, the cell seeded scaffolds were washed three times in PBS and analyzed for cell viability. The viability of cells was qualitatively assessed with a LIVE/DEAD® Viability/Cytotoxicity Kit (Invitrogen) according to the manufacturer's instructions. Fluorescence confocal microscopy (LSM) was used to visualize live (calcein; green) and dead (ethidium homodimer-1; red) cells.

Chondrogenesis of HWJ-MSC seeded on CHAG scaffolds with dynamic compression loading and growth factors

CHAG scaffolds were seeded with 1×10^6 HWJ-MSCs and cultured in Chondrogenic medium as 4 independent groups. The cell seeded scaffolds in Chondrogenic medium were supplemented with ether 1) 10 ng ml^{-1} TGF-β3 (CT-C), 2) 25 ng ml^{-1}BMP-2 (CB-C), 3) 10 ng ml^{-1} TGF-β3 + 25 ng ml^{-1} (CBT-C) or 4) no growth factor (C-C), in the Chondrogenic medium. The day of seeding was counted as day-1, and the cell seeded scaffolds were cultures in 6-well plates till day-7. At day 8 the cell seeded scaffolds were transferred aseptically to the Cartigen C10-12 bioreactor, (Tissue Growth Technologies, USA) and dynamic compression loading was applied at 1Hz, one hour/day, till day 14 for the respective groups. Medium was changed every second day.

DNA, S-GAG quantification

After 14 days of culture, HWJ-MSCs seeded CHAG scaffolds were retrieved and digested individually in papain digest solution (2.5 unit's papain ml^{-1}, 5mM cysteine HCl, 5mM EDTA, in PBS) at 60°C overnight. Digested samples were assayed for total DNA content using Quant-iT™ dsDNA high sensitivity assay kit and for Sulfated Glycosaminoglycan (sGAG) using DMMB assay. All biochemical assays were performed with n=4 independent replicates.

Histological examination of HWJ-MSC seeded CHAG scaffolds

After 14 days of culture, cell seeded CHAG scaffolds were washed with PBS twice and fixed in neutral buffered formalin for 1 hour at room temperature, washed with PBS three times. The cell seeded scaffolds were frozen at-20°C in tissue freezing medium (Leica) and cryosections of 10µm were cut and transferred to micro slides (Himedia) and allowed to adhere for 48 hours at 4°C. Then the slides were stained for deposited sGAG of the ECM with safranin-O-fast green [22] and for cell nuclei and matrix with Haematoxylin and Eosin (H&E). Immunohistochemical Staining (IHC) was performed for collagen-II and nucleus was stained with DAPI and the slides were mounted and viewed on Leica DM4000 microscope.

Gene expression analysis

After 14 days, total RNA was isolated from the cell seeded CHAG scaffolds by using an RNeasy kit and cDNA was obtained from reverse transcription using the QuantiTect Reverse Transcription kit (all from Qiagen, UK). Real-time Quantitative Polymerase Chain Reaction (qPCR) was carried out on a Light Cycler® 96 Real-Time PCR system, using custom made and validated primer sequences for COL2A1, COL1A1, ACAN, COL10A1, SOX6, SOX9 and COL10A1. All qPCR measurements were performed with n=3 independent replicates. qPCR were performed on using Takyon qPCR kit for SYBR assay kit in accordance with the manufacturer's recommendations (Erogentec, UK). The gene expression levels were normalized to the expression of the housekeeping gene GAPDH, VIM and were expressed as fold changes relative to day 0 passaged HWJ-MSC control samples (2h post-seeding). The relative mRNA levels of COL2A1, COL1A1, ACAN, COL10A1, SOX6, SOX9 and COL10A1 were calculated using the ΔΔCt method and the COL2A1/COL1A1 ratio corresponds to the $2^{-\Delta\Delta Ct}$ of each gene.

The following primers were used: COL2A1 (Forward 5'-CTG-GTGAAGAAGGCAAGAG-3'and Reverse 5'-CCATCTTGACCT-GGGAAAC-3'), COL1A1 (Forward 5'-GGATTCCAGTTCGAG-TATGG-3' and Reverse 5'-CAGTGGTAGGTGATGTTCTG-3'), ACAN (Forward 5'-GTGATCCTTACCGTAAAGCC-3' and Reverse 5'-GTCTCATTCTCAACCTCAGC-3'),SOX9 (Forward 5'-GGCCAACCTTGGCTAAAT-3' and Reverse 5'-CACAC-GATTCTCCATCATCC-3'),SOX6 (Forward 5'-GTCCCGTCATGT-CAGTTTAG-3' and Reverse 5'-AACCCTGACCCTGTAAGT-3'), COL10A1 (Forward 5'-CGTATGTGAAGCCTCTCTTG-3' and Reverse 5'-GCAGGACTTCTTTGGTGATA-3'), VIM (Forward 5'-TCACTCCCTCTGGTTGATAC-3' and Reverse 5'-GTCATC-GTGATGCTGAGAAG-3'), GAPDH (Forward 5'-CAAGAGCA-CAAGAGGAAGAG-3' and Reverse 5'-CTACATGGCAACTGT-GAGG -3').

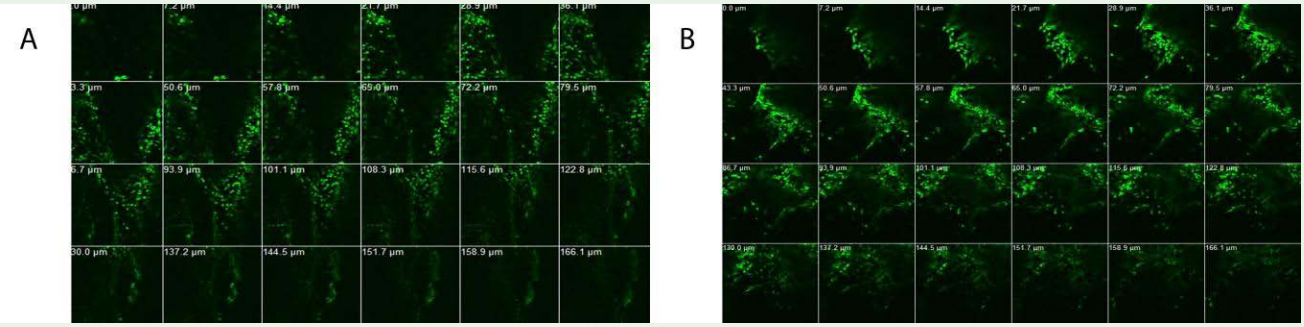

Figure 1: Live dead imaging using confocal microscope at 20X magnification. (A) L929 cells seeded on CHAG scaffolds and cultured for 3 days, (BHWJ-MSC seeded on to CHAG scaffolds and cultured for 3 days.

Statistical analysis

All experimental groups had a sample size of at least n=3 for biochemical and qPCR and n=6 for mechanical property analyses. Data are presented as average ± standard deviation. Statistical significance was determined by performing one-way ANOVA with a significance accepted at p-value < 0.05.

Results and Discussion

CHAG scaffold preparation and characterization

CHAG scaffolds were prepared and characterized as communicated earlier (under review JBMR-A). Briefly, the scaffolds have a compression modulus of modulus of 1.33MPa, which is ideal for cartilage [23]. FTIR spectrum did not show any significant shift in the absorption peaks of chitosan or agarose, owing to a blend and similarly had glass transition temperature was 92.45°C which lies between that of chitosan (86.64°C) and agarose (93.90°C). The cross sections of the scaffolds were analyzed using SEM. The SEM images revealed the evidence of pore interconnectivity and the organized and uniform pores in the scaffold would promote uniform neo-cartilage formation [24]. Also µCT analysis revealed that more than 80% of the pores were between 75µm to 300µm which would be beneficial for the growth of neo cartilage tissue. These properties of CHAG scaffolds were comparatively better than Hyalograft C autograft having a pore size of 200µm and Novocart 3D having a pore size of 10-100 µm, which could support better cell infiltration and neo-cartilage formation [25]. The CHAG scaffolds had water uptake ability in the range of 14.5±3.6 times the dry weight of the scaffold which would help in being a better scaffold. The CHAG scaffolds had a degree of degradation of 18% over a period of six months, hence would provide a stable support for the newly formed tissue.

Cell infiltration

The cell infiltration of HWJ-MSC and L929 cells on the scaffolds were studied using confocal microscopy. The cross section of the cell seeded scaffold after 3days of culture were stained with live/dead stain which showed a very good cell attachment. The confocal images showed the presence of live cells into the deep layers of the scaffolds unlike the Chondro-Gide graft having minimal cell, deep into the scaffolds [25]. This shows that both HWJ-MSC and L929 cells (Figure 1) could infiltrate into the scaffolds [26,27].

Chondrogenesis of HWJ-MSCs seeded on CHAG scaffolds with the aid of dynamic compression loading

There are many previous reports that shows dynamic compression loading would enhance the Chondrogenic potential of cells seeded onto scaffolds [20]. Previous reports suggests, that there is a substantial increase in the Proteoglycans synthesis upon applying dynamic compression loading on adult chondrocytes seeded on agarose constructs and also increased the young's modulus of the chondrocytes seeded agarose constructs [18,19,28]. We wanted to study the effect of dynamic compression loading in the chondrogenesis of mesenchymal stem cell (HWJ-MSCs) seeded on to the CHAG scaffolds and also understand whether the cells seeded scaffold would withstand dynamic compression loading. CHAG scaffolds were seeded with HWJ-MSCs $1x10^6$ cells per scaffolds and cultured in Chondrogenic medium supplemented with or without growth factors (BMP-2, TGFβ3 or BMP-2+TGFβ3) for 7 days in 6-well culture plate. Then to mimic the in-vivo microenvironment of the native cartilage they were cultured in Cartigen bioreactor and dynamic compression loading was applied from 8 to 14 days, such that the scaffolds received 10% strain, at 1Hz frequency, for 1 hour/day.

The morphology of the HWJ-MSCs seeded on to the CHAG scaffolds were analyzed using SEM (Figure 2). The cell seeded scaffolds were intact and cells were having rounded morphology similar to chondrocytes embedded in the ECM secreted by it. The cell seeded scaffolds which received BMP-2+TGFβ3 and dynamic compression loading spread well and covered the entire pores of the scaffolds (Figure 2D). The cell seeded scaffolds had good cell attachment and produced GAGs. The total GAGs content estimated using DMMB assay and expressed in terms of GAGs/DNA content, is shown in figure 2E, 2F.The cell seeded scaffolds supplemented with both BMP-2+TGFβ3 and dynamic compression loading had produced more GAGs than the other which either received BMP-2, TGFβ3 or no growth factor supplements (Figure 2A, 2B, 2C) along with dynamic compression loading. The scaffolds which received only dynamic compression loading had produced more GAGs (Figure 2E, 2F) than the one which received dynamic compression loading and supplemented with either of the growth factors (BMP-2 or TGFβ3). This suggests that dynamic compression loading alone has a positive effect on the chondrogenesis of HWJ-MSCs seeded on CHAG scaffolds.

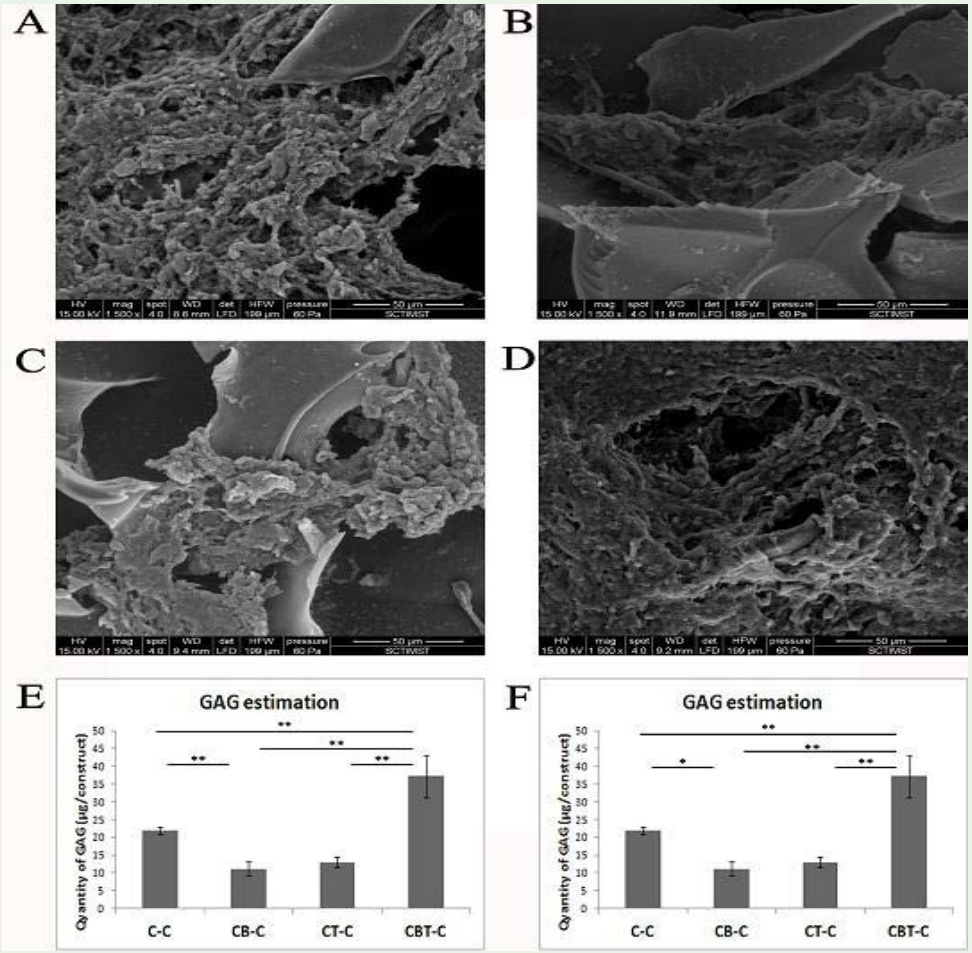

Figure 2: Chondrogenesis of HWJ-MSCs seeded on to the CHAG scaffolds with dynamic compression loading. SEM images of HWJ-MSCs cultured in chondrogenic medium for 14 days supplemented with (A) No growth factors, (B) BMP-2, (C) TGFβ3, (D) BMP-2+TGFβ3 and under the influence of dynamic compression loading, (E) GAG estimated per construct, (F) Estimated GAGs expressed in terms of GAG/DNA content. . C-C: chondrogenic medium, CB-C: chondrogenic medium + BMP-2, CT-C: chondrogenic medium + TGFβ3, CBT-C: chondrogenic medium+BMP-2+TGFβ3. Values are represented as means ± SD (n=3). *P < 0.05, **P < 0.01.

The effect of dynamic compression loading and medium supplementation with growth factors on the chondrogenesis of HWJ-MSCs seeded on CHAG scaffolds was studied. The gene expression of the chondrogenic markers, COL2A1, ACAN SOX6 and SOX9 is shown in figure 3. Cell seeded scaffolds cultured in chondrogenic medium supplemented with BMP-2 and dynamic compression loading showed enhanced gene expression of ACAN and SOX9 (Figure 3C,3D), while the one which had both BMP-2+TGFβ3 had more COL2A1(Figure 3A). BMP-2 stimulates MSCs condensation and enhances chondrocytes proliferation and matrix synthesis [29, 30]. The differentiation index, ratio of COL2A1/COL1A1 was almost 1.5 or more in all the groups. The cell seeded scaffolds which received dynamic compression loading and BMP-2 was higher than the others. This also showed that the CHAG scaffolds supported chondrogenic differentiation of HWJ-MSCs under the influence of dynamic compression loading. Medium supplementation with either BMP-2 or TGFβ3 alone induced chondrogenesis, but simultaneous dynamic compression loading suppressed the effect, while dynamic compression loading alone induced chondrogenesis in the absence of growth factors [31]. The up regulation of SOX6 and SOX9 was not observed in our experiments unlike the native tissue suggesting that endogenous SOX signaling in the HWJ-MSCs is enough for matrix production[32]. The gene expression levels of COL1A1 and COL10A1 were very low in each of the groups indicating that HWJ-MSCs had undergone chondrogenesis and remaining chondrocytes like differentiated state [31]. Ando et al., (2009) have reported that mechanical loading of rat chondrocytes seeded in three dimensional scaffolds increased the secretion of collagen type-II, while lowering type-I collagen. Dynamic loading on canine chondrocytes seeded in agarose hydro gels increased the stiffness of the constructs than the non loaded constructs [28].

Histology staining of the cell seeded scaffolds cultured under the influence of dynamic compression loading is shown in figure 4.HWJ-MSCs, seeded on to the CHAG scaffolds were cultured in chondrogenic medium, supplemented with or without the growth factors BMP-2 or TGF-β3 or both for 14 days such that all received cyclic dynamic compression from day 8-14.H&E staining showed that the HWJ-MSCs attached well on the CHAG scaffolds and produced ECM.The HWJ-MSCs seeded on to the CHAG scaffolds

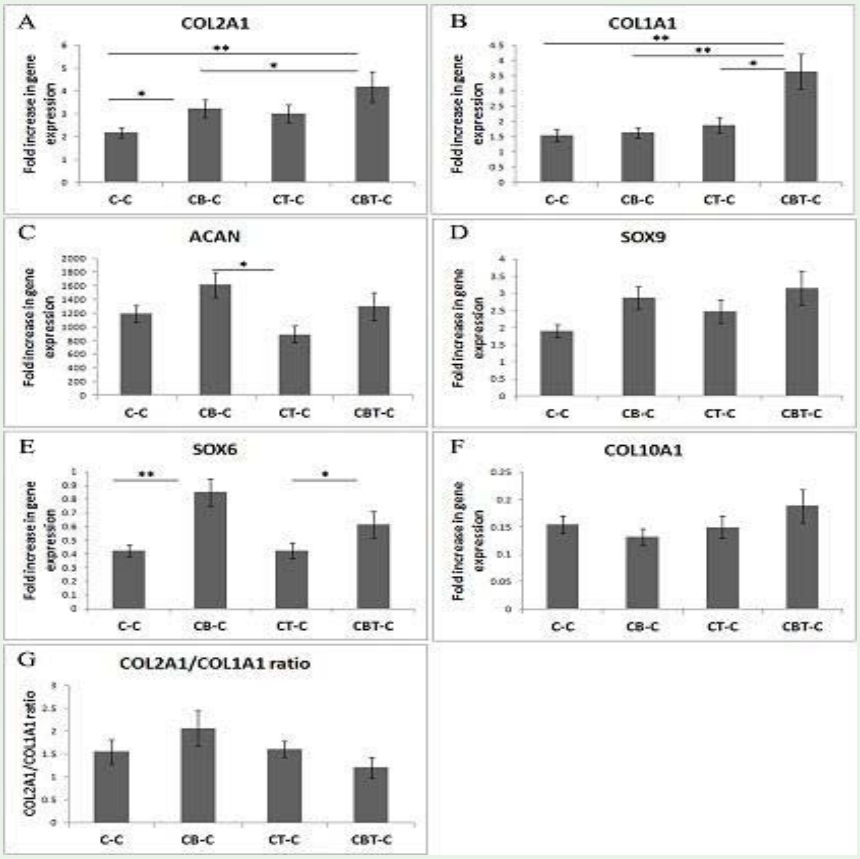

Figure 3: Gene expression of (A) COL2A1, (B) COL 1, (C) ACAN, (D) SOX9, (E) SOX6 and (F) COL10A1 for HWJ-MSCs seeded on to CHAG scaffolds and cultured for 14 days in chondrogenic medium with dynamic compression loading as measured by qPCR and analyzed using the ΔΔCT method, represented as a fold difference relative to undifferentiated HWJ-MSCs (calibrator) prior to seeding and normalized to GAPDH, VIM. (G) COL2A1/COL1A1 ratio was high. C-C: chondrogenic medium, CB-C: chondrogenic medium+BMP-2, CT-C: chondrogenic medium+TGFβ3, CBT-C: chondrogenic medium+ BMP-2 + TGFβ3. Values are represented as means ± SD. (n = 3). *P < 0.05, **P < 0.01.

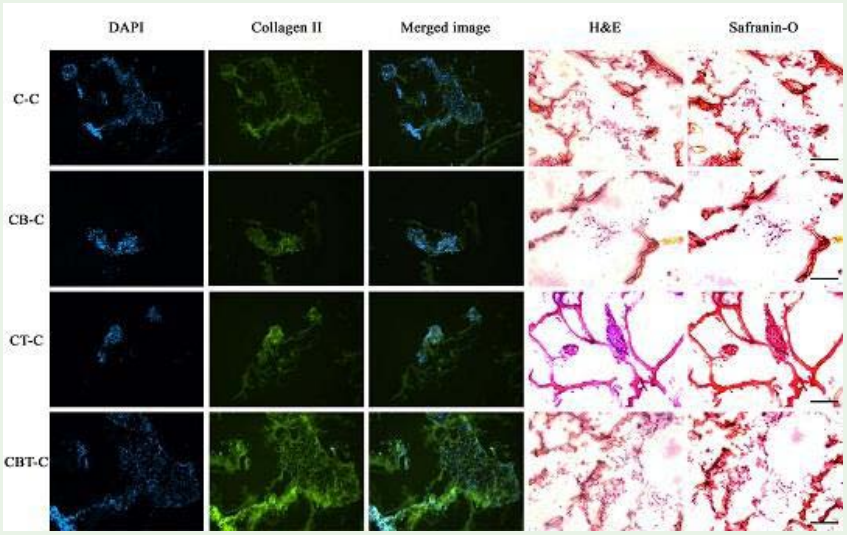

Figure 4: Histological examination of HWJ-MSCs seeded on to CHAG scaffolds and cultured for 14 days in chondrogenic medium with dynamic compression loading. The constructs were immune-stained with DAPI, Palloidin conjugated with Alexa flor 488, merged image, and stained with haematoxylin and eosin (H&E), Safranin-O for sGAG, respectively, from left to right. . C-C: chondrogenic medium, CB-C: chondrogenic medium+ BMP-2, CT-C: chondrogenic medium + TGFβ3, CBT-C: chondrogenic medium+BMP-2+TGFβ3. All scale bars represent 100μm.

and cultured with supplementation of either BMP-2 or TGF-β3 in the chondrogenic media formed larger cluster or safranin-O staining showed that the cells are embedded in the matrix produced by it is of cartilaginous type. Whereas the cell seeded scaffolds which were subjected to dynamic compression loading and received no growth factor supplementation or both BMP-2 + TGF-β3 supplementation have spread well and covered the pores was evident in the H&E staining. Safranin-O staining revealed the cells seeded scaffolds which received both BMP-2 and TGF-β3 along with dynamic compression loading had produced more cartilaginous matrix than the other groups confirm the positive effect of dynamic compression loading [20,33-36] in the differentiation of HWJ-MSCs to a chondrogenic lineage like phenotype. Immunostaining for actin showed the cells grown covering the pores and integrated cell morphology. The cell seeded constructs that either received no growth factors or both BMP-2 + TGF-β3 supplementation had clear demarcation which covered the pores. The cartilage specific gene expression COL2A1 and differentiation index, ratio of COL2A1/COL1A1, enhanced accumulation of GAGs implicates the matrix produced by the cells are cartilaginous. This also demonstrates the positive effect of dynamic compression loading on HWJ-MSCs seeded on to CHAG scaffolds to differentiate to chondrogenic lineage and produce ECM.

Conclusion

We have developed CHAG scaffolds with high porosity, cell adhesion and infiltration, resilient enough to withstand dynamic compression loading, tailor made for cartilage tissue engineering. It has very good cell attachment for L929 cells and HWJ-MSCs. The chondrogenesis of HWJ-MSCs seeded on to CHAG scaffolds with the influence of dynamic compression loading was studied. The scaffolds supported dynamic compression loading and enhanced the chondrogenesis evidenced by the GAGs accumulation. In the cell seeded scaffolds which were supplemented with BMP-2+TGF-β3 and subjected to dynamic compression loading, total GAG content and GAG/DNA content was significantly higher, even though the gene expression of COL2A1 and ACAN was lower on day 14. Very low levels of gene expression of COL1A1 and COL10Al indicate that the HWJ-MSCs are differentiated to a matrix producing chondrocytes like cells. Our study demonstrates the potential of the CHAG scaffolds which could be used in combination with dynamic compression loading to generate the microenvironment so as to generate functional tissue engineered construct. Further detailed studies need to be carried out to optimize the use of appropriate growth factor supplementation and loading regime.

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