PREPARATION OF COLLAGEN PEPTIDE CROSS-LINKED 3D GELLAN GUM HYDROGEL SCAFFOLD FOR IN-VITRO ARTICULAR CHONDROCYTE CULTURE AND CARTILAGE TISSUE REGENERATION

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Abstract

Human Articular Cartilage is an avascular structure, which, when injured, has very slow healing capacity as compared to other tissues. Therefore, innovative ideas like tissue engineering have been a promising approach to facilitate regeneration of hyaline-like articular cartilage to avoid continued pain, arthrodesis, or joint arthroplasty. Obtaining appropriate cells for implantation; directing the development of those cells on a chondrogenic pathway using growth factors and/or cytokines; supporting the growing cells on a three-dimensional matrix; and having that matrix remain in the defected cartilage, at least until the cartilage is healed are the key factors for successful tissue engineering. In this respect, nature-derived hydrogel scaffolds have huge potential for cartilage tissue engineering, mimicking the native cellular microenvironment. This project demonstrates that the three-dimensional (3D) scaffold made up of a polysaccharide called gellan gum and collagen peptide can be used for in vitro culture of chondrocytes. The hydrogel of two different compositions were prepared out of which one was gellan gum hydrogel and the other one was collagen peptide crosslinked gellan gum hydrogel to be used as a scaffold. To compare the mechanical properties, swelling tests were performed and FTIR analysis was done to show the composition and crosslinking in the hydrogel. Then, human articular chondrocytes were isolated and encapsulated in each hydrogel and were cultured. Lastly, MTT assay was done to analyze cell viability and proliferation in each hydrogel which showed there was good proliferation of chondrocytes within the hydrogels.

Keyword: 3D, scaffold, gellan gum, polysaccharide, MTT assay, collagen peptide, chondrocytes, culture

1.INTRODUCTION

Chondral and osteochondral injuries are commonly seen in today's clinical practice. It is estimated that about 10% of the world's population aged 60 years or older have significant clinical problems that could be attributed to osteoarthritis (OA). Articular cartilage provides an ultimate low-friction gliding surface, which none of the artificial constructs have been able to replace successfully. Retrospective review of the knee arthroscopies has revealed an underestimated incidence of this complex problem. Cartilage injuries in the knee joint if left untreated lead to pre-mature early arthritis and affect the activities of daily living. Various different treatment methods of cartilage regeneration have shown encouraging results, but unfortunately none has proved to be the ultimate solution. Up until 1990, marrow stimulation techniques were routine form of management for chondral defects. However, ever since autologous chondrocyte implantation was successfully introduced in humans, it has provided a new dimension for the treatment of chondral defects [1]. For the past three centuries, physicians and scientists have sought several different ways to repair or regenerate articular surface of synovial joint following traumatic damage or

degeneration of the cartilage. A typical tissue response to injury follows a cascade of necrosis, inflammation, repair and scar remodeling. Vascular phase of this cascade is the most important determinant of healing. Hyaline cartilage, being avascular structure, lacks an ability to generate this vital response. Thus, after any mechanical insult or damage, the intrinsic reparative ability of cartilage is very low. Healing of the cartilage defect means restoring structural integrity and function of the damaged tissue. Gellan Gum (GG) has been recently proposed for cartilage tissue engineering applications. GG hydrogels are produced by physical cross-linking methods induced by temperature variation or by the presence of divalent cations. Collagen peptides have also shown a good response to cartilage tissue regeneration [2][3]. Hence, this study presents a new class of GG hydrogel cross linkable by both physical and chemical mechanisms where collagen peptides are incorporated in the GG chain leading to the production of a collagen peptide crosslinked Gellan Gum hydrogel with highly tunable physical and mechanical properties. The chemical modification is confirmed by Fourier Transform Infrared Spectroscopy (FTIR) [4]. The swelling kinetics of the hydrogel and hydrolytic degradation rate is dependent on the cross-linking mechanisms used to form 2 the hydrogels [5]. Several methods have been introduced for the chondrocyte isolation from different tissue parts and organisms over the last decades. In this study, Primary human adult articular chondrocytes are isolated from the surgical waste obtained during total knee arthroplasty (TKA) by enzymatic digestion [6]. Thus, isolated chondrocytes are encapsulated in the hydrogels, cultured in vitro, and analyzed for cell viability and ECM production [7].

2.METHODOLOGY

2.1 Preparation of hydrogel

2.1.1 Preparation of gellan gum hydrogel

0.9 gram of Gelrite (Oxford Lab Fine Chem LLP) was measured and was dissolved in 100 ml of deionized water under constant stirring on a magnetic stirrer at 50°C for 10 minutes. The solution was progressively heated to 90°C, under which complete and homogeneous dispersion of the material was obtained after 30 minutes. Afterwards, 0.03% (w/v) calcium chloride was added to the solution. After dissolution the temperature of the solution was progressively decreased to 45°C. Then, the plain GG hydrogel was produced by casting the solution into petri dish and 24 well plates and allowing the temperature to cool to room temperature.

2.1.2 Preparation of gellan gum and collagen peptide hydrogel

0.9 gram Gelrite and 0.9 gram collagen peptide was measured and dissolved in 100 ml of deionized water respectively under constant stirring on a magnetic stirrer at 50°C for 10 minutes. The solution was progressively heated to 90°C, under which complete and homogeneous dispersion of the material was obtained after 30 minutes. Afterwards, 0.03% (w/v) Calcium chloride was added to the solution. After dissolution the temperature of the solution was progressively decreased to 45°C. Then, the GG and collagen peptide hydrogel was produced by casting the solution into petri dish and 24 well plates and allowing the temperature to cool to room temperature.

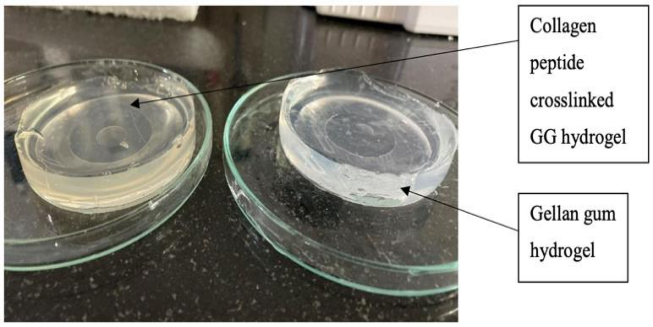

Figure 1: Gellan gum and collagen peptide cross-linked gellan gum hydrogels

2.2. Swelling test

To study the swelling kinetics of the developed hydrogels, three solutions with different ionic content were used: distilled water (no ions); phosphate buffered saline (PBS) Dulbecco's Modified Eagle's medium (DMEM). Hydrogel samples at 0.9% (w/v) were immersed in 2 mL of each solution at 37°C. At different time points, the hydrogels (n=3) were removed from the solutions and were quickly blotted on a filter paper. Their wet weight was measured (wt) and compared to

the initial wet weight (w0). The swelling ratio (Sk) was defined according to equation below:

$$S_k(\%) = \left(\frac{w_t - w_0}{w_0}\right) * 100$$

2.3. FTIR test

In order to characterize the composition of hydrogel and its constituent functional groups, FTIR test was performed on samples of GG only and GG crosslinked with collagen peptide at Central Department of Chemistry, Tribhuvan University.

2.4. Isolation of chondrocytes

Tissues from lateral and medial femoral condyles, and tibial plateau, are routinely removed during total knee arthroplasty. After surgery, leftover osteochondral tissue was immediately transferred to cell culture laboratory in PBS under sterile conditions. Collagenase B (Roche Diagnostics Gmb H, Germany) solution was prepared by dissolving 30 mg of collagenase B powder in 15 ml DMEM.

Chondrocytes were isolated by enzymatic digestion. For this the human articular cartilage, free from all surrounding tissue, was placed in a Petri dish containing sterile PBS and cut into small pieces. The pieces were then washed in sterile PBS and immersed in trypsin–ethylenediaminetetraacetic acid solution, and incubated for 30min at 37°C. Afterwards, Trypsin was removed and the tissue pieces was washed with basic DMEM. Then, previously made collagenase-B (2mg/mL) in basic medium was added, and the mixture was incubated for approximately 12hr. at 37°C. The digested tissue and cell suspension solution was centrifuged at 4000 rpm for 7min and the supernatant were discarded. The cell pellet was washed with PBS and centrifuged again under the same conditions. Cells were again centrifuged, the supernatant were removed, and resuspended in expansion medium consisting of DMEM containing 10,000 units/mL penicillin or 10,000µg/mL streptomycin, 20mM L-alanyl glutamine, 1× minimum essential medium (MEM) nonessential amino acids, and 10% (v/v) fetal bovine serum. Human articular chondrocytes will be plated onto tissue culture flasks and incubated at 37°C in a humidified atmosphere of 5% CO2 in air for expansion.

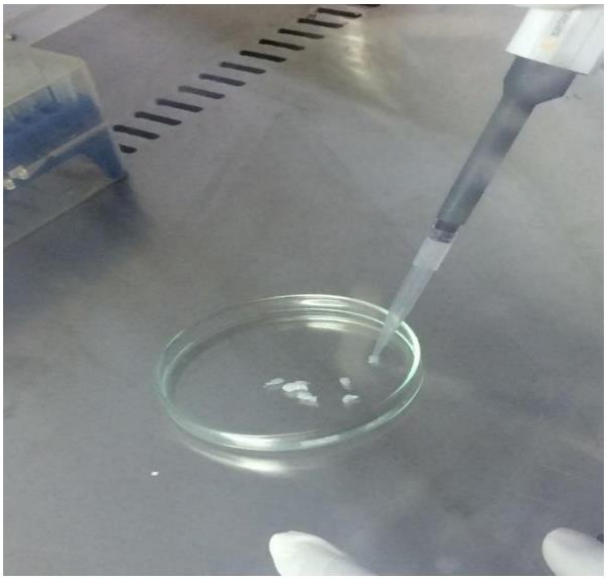

Figure 2: Human articular cartilage in a petri dish containing PBS

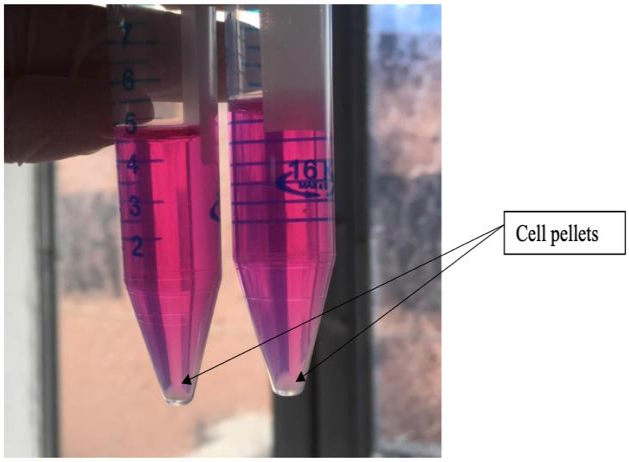

Figure 3: Cell pellet after centrifugation

2.5. In vitro cell encapsulation

The human articular chondrocytes were expanded and encapsulated at passage 1 in gellan gum hydrogel and collagen peptide crosslinked gellan gum hydrogel.

For the preparation of gellan gum hydrogel, 0.1 gram of gellan gum powder was dissolved in 50 ml of sterile distilled water under constant stirring at room temperature. The solution was progressively heated to 900°C and kept at this temperature for 20-30 minutes. A sterile CaCl2 solution was added to obtain a final

concentration of 0.015% (w/v). The temperature was progressively decreased to 420C and stabilized at this value for later use under constant stirring.

Similarly, collagen peptide crosslinked gellan gum hydrogel was prepared by dissolving 0.1 gram of gellan gum powder along with 0.1 gram of collagen peptide powder in 50 ml of sterile distilled water under constant stirring at room temperature. The solution was progressively heated to 90°C and kept at this temperature for 20-30 minutes. A sterile CaCl2 solution was added to obtain a final concentration of 0.015% (w/v). The temperature was progressively decreased to 42°C and stabilized at this value for later use under constant stirring.

Human articular chondrocytes were detached by trypsinization. The media was discarded and then the chondrocytes were washed with Phosphate Buffer Saline (PBS) 2-3 times until zero trace of media was seen. Then, 2 ml of trypsin was added and the flask was incubated for 3-4 minutes. The flask was checked for cell detachment and then 4 ml of media was added. The solution was transferred to two falcon tubes and centrifuged at 4000 rpm for 4 minutes to form cell pellets. Two different cell pellets were obtained and each of them were resuspended in gellan gum hydrogel and collagen peptide crosslinked gellan gum hydrogel discs containing chondrocytes were produced by casting this mixture into 24 well plates and then allowing it to rest at room temperature for 1-2 minutes to form a solid gel. The discs were then cultured in the expansion medium for 7 days.

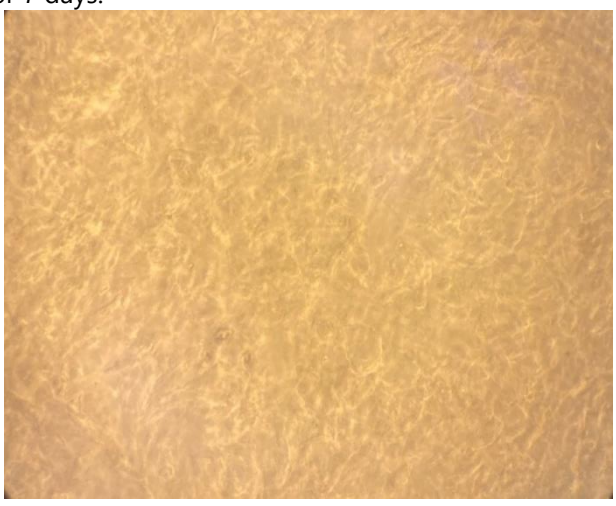

Figure 4: Microscopic view of hydrogels without cells

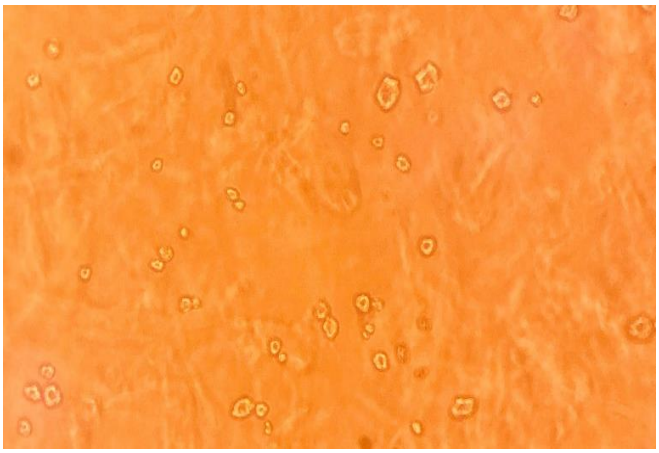

Figure 5: Microscopic view of cell laden hydrogel

2.6. Cell proliferation test

2.6.1 Preparation of MTT reagent solution

The solution was made for 96 wells with each well receiving 40µL MTT reagent solution. So, 3ml MTT assay reagent solution was made. For this, the reagent was first weighed and 0.015 gram was taken in a falcon tube. 3 ml of distilled water was added to the tube and the mixture was kept in vortex mixer for thorough mixing. As the solution was light sensitive, it was wrapped in aluminum foil and kept for further use.

2.6.2 MTT assay

The cells encapsulated and cultured in gellan gum hydrogel and collagen peptide crosslinked gellan gum hydrogel prior 3 days and 5 days were transferred from the 24-well plate into new 96 well plate with proper marking to differentiate each type of hydrogel. The negative control was taken as hydrophobic plastic, gellan gum hydrogel and collagen peptide crosslinked GG hydrogel. Then, 200µL media was added to each well. After that, 40µL MTT assay reagent was added to each well and the 96 well plate was completely covered by paraffin wax and placed in the incubator for 4 hours at 37°C. After that the 96 well plate was taken out of the incubator and 100µL DMSO was added to each well which was again incubated for 15 minutes. After that, the absorbance of solution present in each well was recorded in absorbance microplate reader at 595 nm.

3.RESULTS

3.1 Swelling capacity test

3.1.1. Swelling capacity of hydrogels in distilled water

The results from the experiment performed on 6 samples among which 3 of them were gellan gum hydrogel and 3 of them were collagen peptide crosslinked gellan gum hydrogel showed that gellan gum hydrogel had 465.67, 541.32, 544.68, 548.3, 551.24, 551.21, 550.78, 551.45 % when dissolved in distilled water for 0.5, 1, 1.5, 5, 10, 15, 20, 24 hours respectively. Similarly, gellan gum and collagen peptide hydrogel had 896.41, 899.78, 1007.01, 1173.32, 1178.94, 1179.43, 1179.42, 1179.44% when immersed in distilled water for 0.5, 1, 1.5, 5, 10, 15, 20, 24 hours as shown in figure 6.

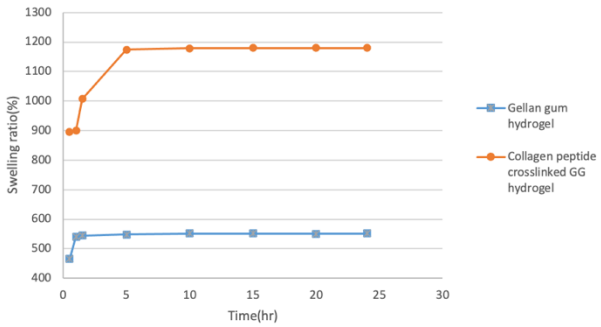

Figure 6: Comparison of the swelling kinetics between GG hydrogel and collagen peptide cross-linked GG hydrogel in distilled water

3.1.2. Swelling capacity of gellan gum hydrogel in PBS

The results from the experiment performed on 6 samples among which 3 of them were gellan gum hydrogel and 3 of them were collagen peptide crosslinked gellan gum hydrogel showed that gellan gum hydrogel had 133.96, 134.72, 140.53, 141.55, 138.32, 137.61, 136.78, 135.41 % when dissolved in PBS for 0.5, 1, 1.5, 5, 10, 15, 20, 24 hours respectively. Similarly, gellan gum and collagen peptide hydrogel had 327.27, 354.54, 345.45 343.39, 341.07, 340.43, 338.98, 335.36% when immersed in PBS for 0.5, 1, 1.5, 5, 10, 15, 20, 24 hours respectively as shown in figure 7.

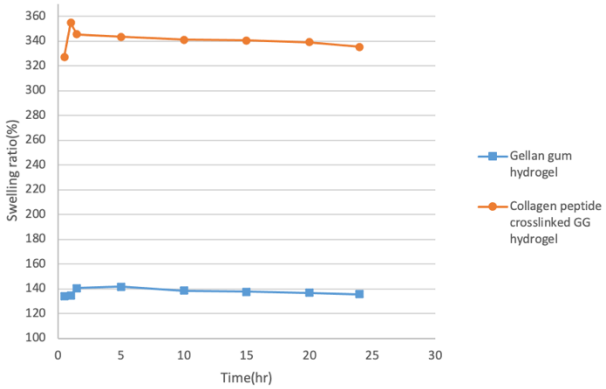

Figure 7: Comparison of the swelling kinetics between GG hydrogel and collagen peptide cross-linked GG hydrogel in PBS

3.1.3. Swelling capacity of gellan gum hydrogel in DMEM

The results from the experiment performed on 6 samples among which 3 of them were gellan gum hydrogel and 3 of them were collagen peptide crosslinked gellan gum hydrogel showed that gellan gum hydrogel had 92.51, 94.11, 94.17, 95.27, 93.63, 90.26, 89.42 and 88.67% when dissolved in DMEM for 0.5, 1, 1.5, 5, 10, 15, 20, 24 hours respectively gellan gum and collagen peptide hydrogel had 264.7, 273.61, 278.56, 275.34, 271.11, 268.84, 265.62% when immersed in PBS for 0.5, 1, 1.5, 5, 10, 15, 20, 24 hours respectively as shown in figure 8.

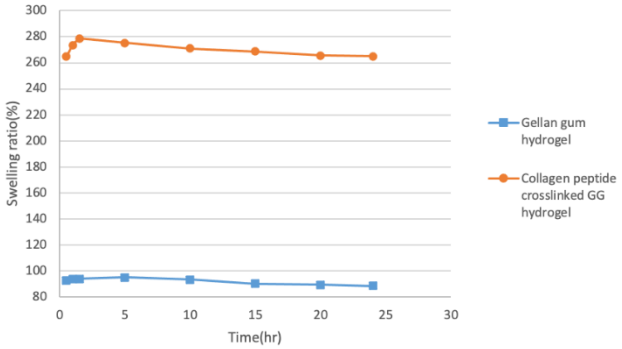

Figure 8: Comparison of the swelling kinetics between GG hydrogel and collagen peptide cross-linked GG hydrogel in DMEM

3.1.4. FTIR test

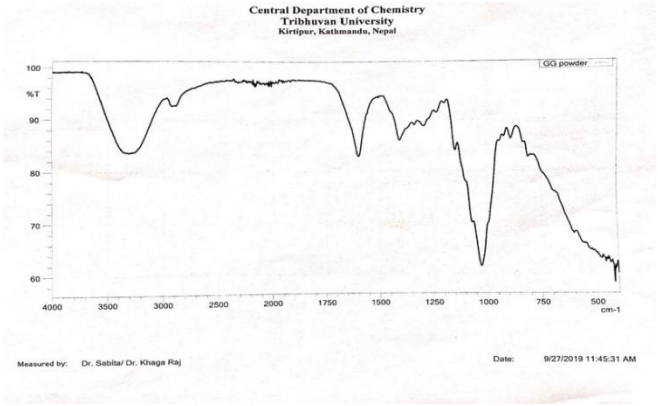

Figure 9: FTIR graph of gellan gum powder

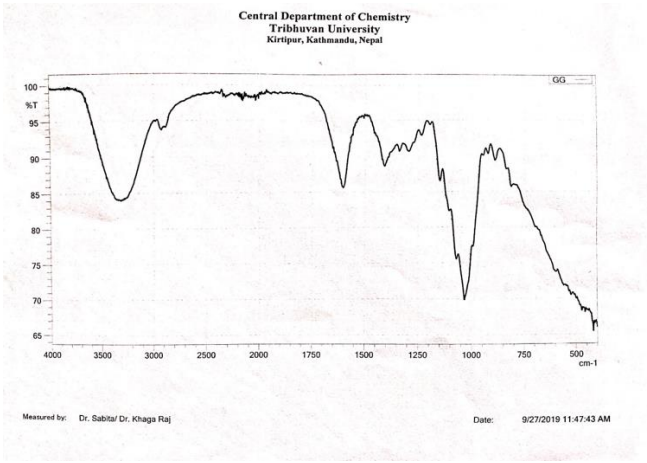

Figure 10: FTIR graph for gellan gum hydrogel

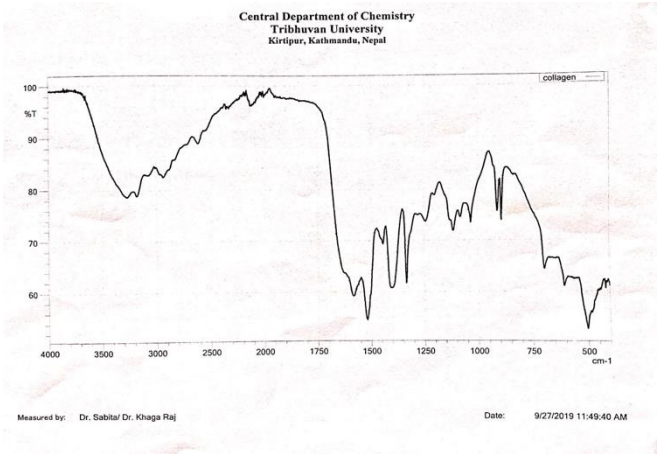

Figure 11: FTIR for collagen peptide powder

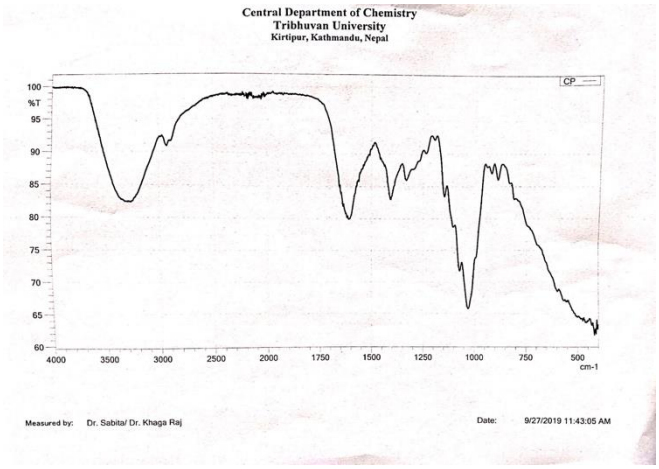

Figure 12: FTIR graph for gellan gum with collagen peptide

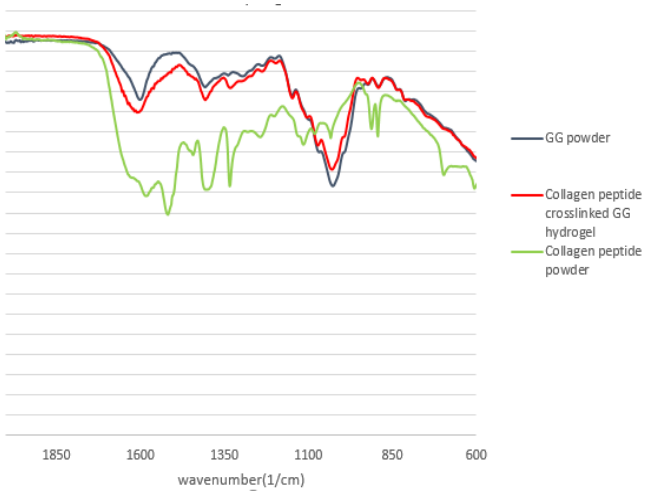

Figure 13: FTIR spectra of GG powder, collagen peptide powder and collagen peptide crosslinked GG hydrogel

The FTIR spectrum obtained for Gellan gum powder, Gellan gum hydrogel, collagen peptide powder and collagen peptide crosslinked GG hydrogel are shown in figure (9-13). Based on the spectrum analysis of collagen peptide crosslinked GG hydrogel broad absorption band at 3250-3500 cm-1 were observed indicating the presence of O-H, hydroxyl stretching. The peaks at the range of 1725-1750 cm-1 attributed to the ester bond and the peaks at 1650 cm-1 demonstrated the presence of peptide bond (C=O).

3.2. Isolation of primary chondrocytes

As mentioned in the Materials and methods section, the full-thickness cartilage was obtained from the femoral condyle of an arthritic knee during knee arthroplasty (TKA) performed at the National Academy of Medical Sciences, Kathmandu. TKA is a common procedure at the mentioned hospital. Since the removed cartilage tissue is considered surgical waste, this presents a reliable and continuous source for isolation of primary chondrocytes.

The primary chondrocytes were isolated as described in the Materials and methods section. During their cultivation, their morphology and proliferation were regularly observed using inverted optical microscopy as shown in figures below. This initial examination was performed to follow possible morphological changes in the cell shapes, which would indicate possible dedifferentiation.

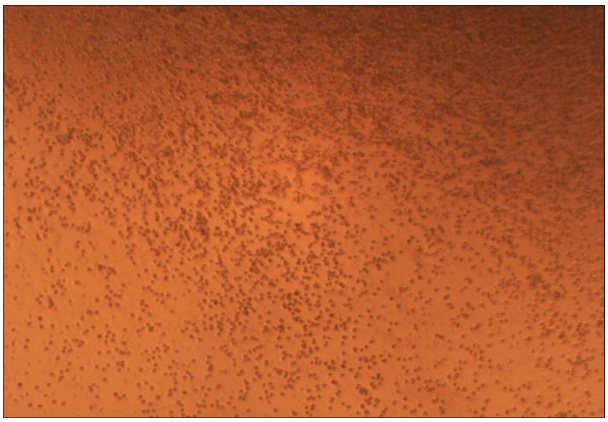

Figure 14: Isolated human chondrocytes observed under microscope 4x

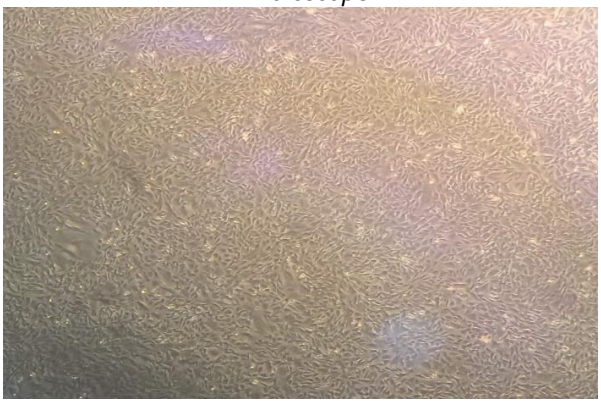

Figure 15: The primary human chondrocyte culture in a monolayer after 2 days under 10x

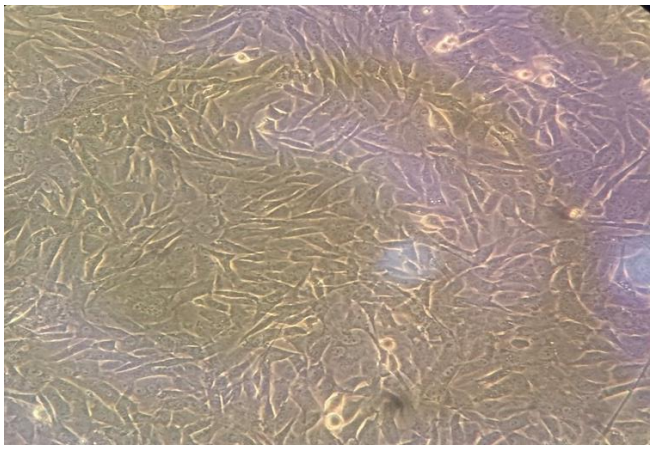

Figure 16: Dedifferentiation of chondrocytes into fibroblast like cells after 2nd passage observed under microscope at 20x

3.3. Cell proliferation

MTT assay was used to determine the cell viability and proliferation inside gellan gum hydrogel and collagen peptide crosslinked GG hydrogel. Formation of formazan was observed in each well containing cell laden hydrogel indicating the presence of live cells inside the hydrogels. After comparing the results by performing ANOVA test, a significant difference was found between the absorbance between 3 & 5 days. Also, the absorbance of the cell laden collagen peptide crosslinked hydrogel was found to be slightly more than that of cell laden gellan gum hydrogel.

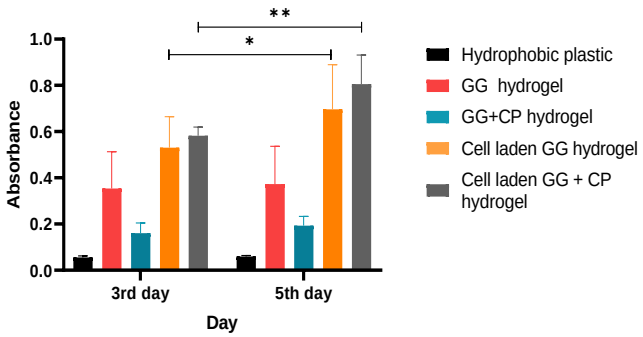

Figure 17: Comparison of cell proliferation through absorbance reading

4.DISCUSSION

GG is capable of physical gelation. As the temperature of a solution of the GG in water is decreased, GG chains

undergo a conformational thermo reversible change from random coils to double helices. Then, the aggregation of the double helical elements occurs to form a three-dimensional (3D) network by hydrogen bonding with water. The gelation of GG is strongly affected by the presence of specific cations, namely Na+, K+, Ca2+ and Mg2+ although divalent cations promote more efficient gelation than monovalent cations. Therefore, in the present work CaCl2 is used in 0.03% (w/v) for the formation of a stable hydrogel.

The hydrogel network is a 3D cross linked matrix, allowing it to hold water in its microstructure and swell. This dynamic ability is due to the hydrophilic nature of the hydrogels. The hydrogels need to have sufficient swelling capacity to allow the cells to grow well with sufficient culture medium and should have low rate of degradation. The degree to which the hydrogels swell can have a direct impact on the behavior of cells encapsulated within the scaffold; thus, swelling properties are important to characterize. In this study, the swelling behavior of the GG hydrogels was characterized in deionized water, PBS, and DMEM media. The hydrogels showed the highest swelling percentage when immersed in deionized water for 30 minutes while hydrogels immersed in both PBS and DMEM for 24 hours showed shrinkage due to the presence of cations supporting the past research for all the hydrogel compositions [5].

Till date there has been no study of preparation of GG hydrogel with collagen peptide. In this project, hydrogels were prepared with GG and collagen peptide in aqueous medium as peptides have multiple functions in cartilage tissue engineering, where they function as active molecules, in cell adsorption, as enrichment motifs, and as scaffold[8].

Our results demonstrated that swelling behavior was significantly impacted by the solution in which the samples were immersed to swell and the composition of the hydrogel. We attribute this behavior to the physical properties of the hydrogel matrix. Properties such as permeability and diffusion coefficient affect how certain liquids enter into the matrix. In addition, the tested liquids have different viscosities that would impact how much liquid volume the hydrogel networks can occupy. Deionized water is the least viscous of the solutions tested; therefore, it can diffuse more readily into the hydrogel matrix. PBS has a higher salt concentration and is relatively more viscous than water. As a result, the hydrogels swelled less in PBS than in deionized water. The DMEM media includes salts and proteins and is thereby the most viscous one among the tested liquids. As expected, the hydrogels swelled the least in the DMEM media. Thus, the comparison gave a clear idea about the strength and swelling capacity of the cross-linked hydrogel used to study the 3-D proliferation of chondrocyte cells.

The FTIR spectrum obtained from GG hydrogel indicated the presence of broad O-H stretching and ester bond. The vibrational mode (mostly peptide bond C=O stretch) was obtained near 1650 cm−1. From the FTIR analysis, all of the expected functional groups from the gellan gum and collagen peptide were found present in the collagen peptide crosslinked GG hydrogel samples. Thus, the presence of ester bond of gellan gum and peptide bond of collagen peptide in collagen peptide crosslinked GG hydrogel has proven the incorporation of collagen peptide into gellan gum hydrogels confirming the crosslinking of collagen peptide with gellan gum.

The preparation of methacrylated gellan gum (MeGG) hydrogel was quite problematic as it was very difficult to maintain the pH of MeGG at neutral range. Also, the handling of the methacrylic anhydride requires additional precautions. Due to this reason the MeGG hydrogel couldn't be used for chondrocytes culture.

After the encapsulation of chondrocytes into the hydrogels, the viability and degree of proliferation of the cells in each type of hydrogel were determined using MTT assay which showed that the cell proliferation in each hydrogel increased with time as the absorbance of the cell laden hydrogels at 5th day was more than that at 3rd day. Moreover, the absorbance observed also indicated that the proliferation of the cell in collagen peptide crosslinked gellan gum hydrogel was slightly greater than that in gellan gum hydrogel. This might be due the reason that collagen peptide helps in ECM formation when cultured for longer period of time[9]. Meanwhile, peptides have been applied in a wide range of applications in medicine and biotechnology over the past decade. Therefore, combining cartilage tissue engineering and the use of peptides may create a number of opportunities[8].

5.CONCLUSION

A 3D biomimetic hydrogel culture platform was prepared to systematically examine cartilage

regeneration potential of chondrocytes. The 3D biomimetic hydrogel is made up Gellan Gum and collagen peptide, which provides a physiologically relevant microenvironment for in vitro culture of chondrocytes. The results presented in this study gives an overview about the use of gellan gum hydrogels as a means for 3D cell culture. It also gives an idea that addition of a collagen peptide can enhance the ECM formation within the hydrogel matrix. Moreover, this study provides the easy, simple and cheaper way of chondrocyte isolation from surgical wastes removed during TKA. The results also highlight the degree of proliferation of cells within different scaffolds and provides a co-relation between their mechanical properties and their effect on cell proliferation.

In addition, the scaffold may be potentially used for cell delivery for cartilage repair in vivo. Furthermore, the mechanical properties of the hydrogel can be tuned by different crosslinking agents such as glutaraldehyde, methacrylic anhydride and so on. Cartilage tissue engineered in the scaffold can be evaluated using quantitative gene expression, immunofluorescence staining, biochemical assays, and mechanical testing. Utilizing these outcomes, the differential regenerative potential of chondrocytes, both at the gene expression level and in the biochemical and biomechanical properties of the engineered cartilage tissue can be characterized.

6.ACKNOWLEDGEMENT

We would like to express our sincere gratitude to College of Biomedical Engineering and Applied Sciences (CBEAS) for providing us the opportunity to do this project and providing us with the required provisions. We would like to express our sincere gratitude towards our supervisors Asst. Prof. Saurav Sharma and Asst. Prof. Mishal Pokharel for their supervision, help, and suggestions.

A very special thanks to Annapurna Neurological Research Centre for providing us their lab and all the necessary equipment needed to complete our project. The help and supervision from Mr. Anil Kumar Sah and Mr. Pusp Raj Joshi are gratefully acknowledged. We would like to show our sincere gratitude to Dr. Archana K.C. Dangal from NAMS and Dr. Avinash Chhetri for helping us during out project.

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