Macroporous matrix for cartilage tissue engineering

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Articular cartilage matrix experiences substantial structural, mechanical and molecular changes with ageing, which include surface fibrillation, variation in proteoglycan structure and composition, increased cross-linking of collagen and decreased stiffness along with tensile strength. Altered functionality of chondrocytes leads to changed extracellular matrix (ECM) functionality and to the development of osteoarthritis. Autologus chondrocytes implantation, the only feasible treatment, relies on the delivery of cultured cells. The purpose of our study was to isolate and examine microenvironment required to maintain the cellular phenotype of cartilage cells. Chondrocytes were isolated from full-thickness articular cartilage of the femoral condyles of skeletally mature bovines by sequential enzymatic digestion. Processed cells were cultured in different systems such as tissue culture plates (2D) and three-dimensional (3D) porous collagen matrix and characterized using cell surface antibodies, biochemical analysis and immunohistochemistry along with viability testing. The chondrocytes native matrix is composed of collagen II and proteoglycan macromolecules. Metabolic activity was found high among cells cultured in 3D collagen matrix and high ECM deposition was noticed. Further confirmation done by S-100 protein and various stains indicated that chondrocytes cultured on three-dimensional matrix retained the cellular phenotypes for longer duration and were highly metabolically active in comparison to the 2D control system.

Keywords: Cartilage, chondrocytes, collagen, macroporous matrix, tissue engineering.

CARTILAGINOUS tissues are found all through the body and perform a wide range of mechanical and structural functions. In diarthroidal joints such as the shoulder and knee, articular cartilage and fibrocartilage play an important role in the free movement and load-bearing capabilities of the joints. High levels and complex combinations of compressive, tensile and shear forces are experienced by these tissues during normal activity. However, the unique compositions and structures of the extracellular matrix (ECM) in articular cartilage and fibrocartilage help the tissue to perform normal biomechanical functions. The cartilage ECM is composed of water (68–85%), collagen (10–20%) and proteoglycans (PG; 5–10%)[1], and the interactions between these components gives the tissue its load-bearing capabilities[2,3]. In addition, the organization of the matrix molecules in three zones of the tissue (superficial, middle and deep) results in a depth-dependent variation in material properties that reflect the complex loading environment within the joint. Osteoarthritis (OA) is a degenerative disease of the cartilage involving complete synovial joint, encompassing the cartilage, synovium and underlying bone. The cells in each tissue are independently incapable to initiate and respond to the joint injury, ultimately resulting in cartilage degeneration. The general belief is that the cartilage degeneration in OA is characterized by two phases – first, the biosynthetic phase during which the cells in cartilage, the chondrocytes, attempt to repair the damaged ECM; and second, the degradative phase, during which the activity of enzymes produced by the chondrocytes digests the matrix thereby inhibiting the matrix synthesis and resulting in accelerated cartilage erosion[4,5].

Articular chondrocytes are the resident cells within the articular cartilage which are responsible for the maintenance of ECM. Articular chondrocytes express and synthesize the major ECM components – collagen II and aggrecan. In addition, the cells produce proteolytic enzymes that break down the matrix and contribute to the homeostatic turnover of the tissue[6,7]. Articular chondrocytes are fully differentiated cells and are normally non-proliferative *in vivo*. Although generally considered a single cell type, there are regional variations in the expression levels of the matrix proteins that reflect the differences in matrix composition[8]. *In vivo*, chondrocytes have primarily round morphologies, but rapidly adhere and spread in monolayer culture. These changes in morphology are accompanied by a steady loss of collagen II and aggrecan expression and reduced PG synthesis[9,10]. However, chondrocytes cultured in three-dimensional environments, such as hydrogels or cell aggregates, can maintain their differentiated phenotype for multiple weeks under the *in vitro* conditions. For tissue engineering applications, ideal scaffold needs to provide a biocompatible 3D architecture that mimics microenvironment of cells of the particular tissue type. Besides being biocompatible, scaffold degradation should be in tune with tissue regeneration without any toxic by-product[11].

Glutaraldehyde (25% v/v), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), Dulbecco's Modified Eagle Medium (DMEM), foetal bovine serum, penicillin/streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ammonia persulphate (APS), dimethyl sulphoxide (DMSO), 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI), phalloidin biotin labelled, 1,9-dimethylmethylene blue (DMB) dye and papain were purchased from Sigma. All the chemicals used were of analytical grade.

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Collagen isolated freshly from rat tail standard protocol was processed and lyophilized. The final weight of the polymer was optimized to 3% (w/v) and dissolved in aqueous solution and 0.1% (w/w) cross-linker glutaraldehyde was added along with free-radical polymerization initiators APS and TEMED. This system was frozen at sub-zero temperature for 12 h followed by lyophilization at –50°C overnight. Fabricated gels were examined for pore distribution and interconnectivity using scanning electron and confocal microscopy.

Articular cartilage tissues were sectioned into very fine sections in phosphate buffer saline (PBS, 0.01 M). The minced tissue was digested with DMEM containing 1.5 mg/ml collagenase type II at 37°C overnight in an incubator. Flasks were observed for single cells liberation every hour[11]. Once single cells started to liberate, they were centrifuged and the supernatant was collected. The supernatant was centrifuged at 1500 rpm/10 min and washed with PBS and the collected cells were cultured with fresh DMEM plus heat-inactivated foetal calf serum, 2 mmol/ml L-glutamine, 25 mmol/ml HEPES and 100 units/ml penicillin and streptomycin at 37°C in humidified 5% CO_2. After first passage the cells were trypsinized and cultured on 3D collagen matrix and 2D tissue culture plate and various biochemical analyses were performed at periodical intervals. All experiments were set up in triplicate for obtaining statistically significant readings.

The secretion of PG and collagen by chondrocytes on scaffold is an important parameter and can be measured by indirect quantitative determination of glycosaminoglycan (GAG) concentration with a spectrophotometer using DMB dye. The GAG content was analysed from chondrocytes seeded samples along with the control at every 5th day up to day 25 of cell culture. The amount of sulphated glycosaminoglycan (S-GAG) in the chondrocytes culture was quantified by the digestion of cell culture samples in papain solution, pH 6.5 (2 mg/ml), taken in tubes and incubated overnight at 60°C. The whole digested samples were collected in 2 ml micro-centrifuge tube and centrifuged at 580 *g* for 20 min. The supernatant was mixed with DMB dye solution and allowed to react for 30 min. The solutions were then read for absorbance spectrophotometrically at 524 nm. The concentration of GAG in all the samples was calculated based on a standard curve plotted using chondroitin sulphate[6]. The amount of GAG was measured in mg/ml, which was reported as μg GAG per mg dry wt of the sample.

In brief, the collagen scaffolds were lyophilized and subjected to papain (0.1%) digestion overnight at 60°C. Digested samples were hydrolysed using 1 M HCl at 120°C for 10 h and the contents were transferred to glass scintillation vials and incubated uncapped at 90°C until dry (brown residue at the bottom of the vial). Samples were cooled to room temperature and redissolved in 1 ml PBS (0.1 M, pH 7.4). The dried hydrolysed samples were mixed with chloramines T and *p*-dimethyl amino benzaldehyde in equal proportion and incubated in a water-bath at 60°C for 30 min. The reaction mixture in the vials was allowed to cool to room temperature for at least 10 min and read in a colorimetric plate reader at 540 nm (ref. 7). Hydroxyproline standards were simultaneously used in different concentrations and the same protocol was followed to obtain the standard curve to determine the unknown hydroxyproline concentration.

MTT assay is an indirect way of checking cell growth, proliferation and viability, since the mitochondrial enzymes of the proliferating cells oxidize the MTT solution, giving a typical blue–violet end-product which can be quantified by spectrophotometric analysis. Briefly, the collagen matrix (5 mm height and 12 mm diameter) was saturated in the DMEM medium supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin and 1% nystatin for 2 h into a 24-well tissue culture plate. Primary chondrocytes (0.5 ml) were seeded into each pre-saturated matrix section with cell density of 1.0×10^5 cells/ml. The control for the experiment was set up under 2D conditions with cells seeded on the culture plates. The cell-seeded sections along with controls were incubated up to 21 days at 37°C in a humidified atmosphere with 5% CO_2 environment. At each interval, the culture medium was removed from test wells and gently washed with PBS (0.1 M, pH 7.4). The serum-free culture medium was added into thiazolyl blue (MTT; 0.5 mg/ml) and this solution was added into test well followed by incubation at 37°C for 5 h. The medium was aspirated carefully and 1.5 ml of DMSO was added to each test well to dissolve intracellular formazan crystals to get a blue–violet coloured end-product, which was measured for spectrophotometer absorbance at 570 nm.

The preparation of scaffold was aimed to obtain a matrix with high volume fraction porosity, spongy soft texture but mechanically stable and with high water-absorbing capacity. Collagen-based matrix fabricated by freeze-drying technique was macroporous and interconnected. The pore size was quantified using SEM and confocal-associated software. The confocal was used for scanning the scaffold sample until the resolution is lost and the scanned slice of the scaffold was reconstituted into 3D structure and software was used for generating the pore size data (Figure 1 *a–c*). Cell growth and proliferation on collagen matrix were performed to check the biocompatibility by culturing primary cells of chondrocytes isolated from goat knee. Primary chondrocytes isolated were seeded onto collagen matrix pre-equilibrated in the cell culture medium. The growth of chondrocytes on collagen matrix sections was carried out on 5 mm and 200 μm sections to check cell behaviour. The control experiments were set up by seeding cells on 2D culture plates, which were continuously monitored by microscopic examination. The pores of the matrix were large enough to allow the cells to effectively migrate into the pores and also allow good nutrient supply and metabolic

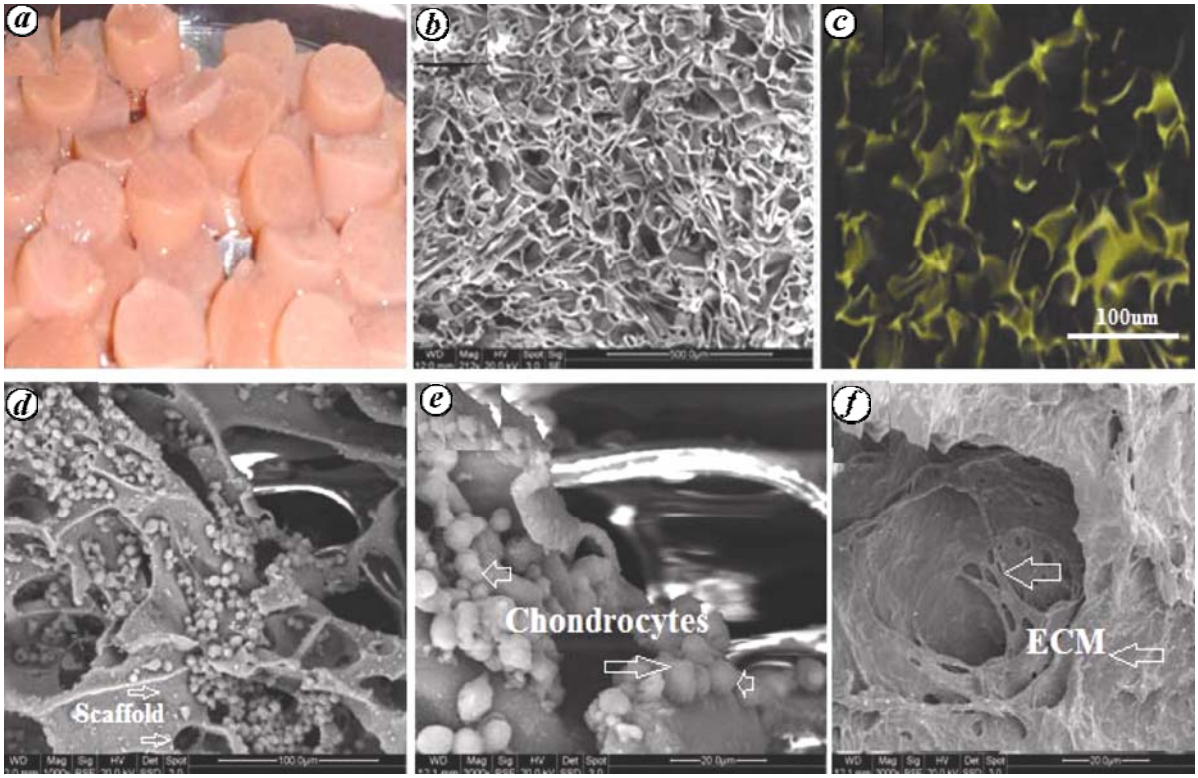

Figure 1. Digital image of collagen scaffold (***a***) showing 3D stability, while scanning electron micrograph image (***b***) showing the macroporous interconnected morphology of scaffolds which is further confirmed using confocal microscopy (***c***). ***d–f***, High chondrocytes attachment on 5th day (***d***), 10th day (***e***) and with extracellular matrix (ECM) deposition (***f***) on scaffold on 20th day of experiment.

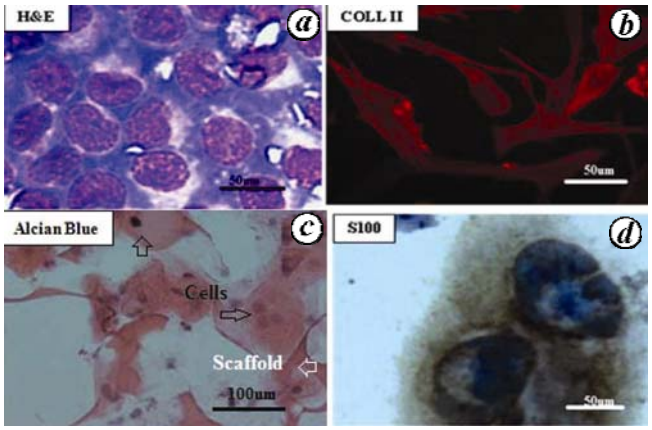

Figure 2 *a–d*. Various immuno-histochemcial analyses performed on chondrocytes cultured on collagen macroporous matrix.

waste removal, essential for effective cell growth. The cells seem well-adhered with uniform distribution and a single cell adhered on the wall of the collagen matrix releasing its own ECM (Figure 1 *d–f*) was checked by various histo and immunochemical analyses. When cells are provided with supportive environment for proliferation, they tend to spread depending upon the surface area. COLL II (Figure 2 *a–c*) production was checked to confirm if chondrocytes were de-differentiating the COLL II production would not be observed while staining. Further confirmation was done by S-100 protein and various other stains showed chondrocytes cultured on three-dimensional matrix retained the cellular phenotypes for longer duration and were highly metabolically active in comparison to 2D control system (Figure 2 *d*).

Chondrocytes are known to lose the phenotype under *in vitro* conditions once they attain confluency[11] due to the synthesis of sulphated-proteoglycans (GAG moiety) leading to a sharp decrease in the pH of the nutrient medium, which increases the chances of cells becoming metabolically inactive or leading to cell death. Different types of staining were performed to check for cellular proliferation along with the ECM component secretion. The nuclei staining DAPI (Figure 3 *a*) confirmed the attachment of chondrocytes to the collagen scaffold and during the following weeks the cells continued to remain active by secreting the native ECM component (Figure 3 *b*). Phalloidin staining was done to check for phenotypic alteration, confirming that the production of COLL II does not result in the change in cell morphology (Figure 3 *c*).

Chondrocytes proliferation and their metabolic activity in collagen scaffolds and 2D system were monitored at regular time intervals up to 25 days of culture by MTT assay. Initial readings showed that the proliferation of

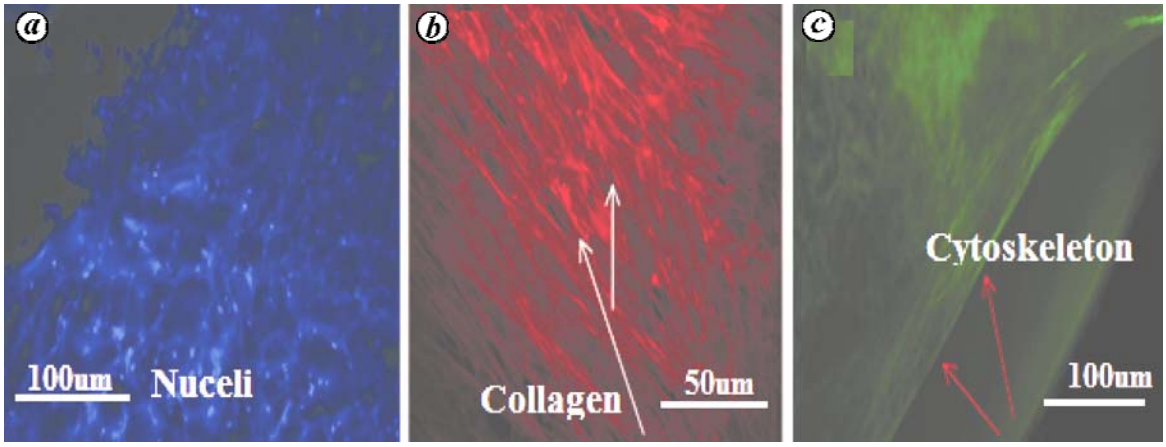

Figure 3. Cytoskeletal staining using phalloidin to check the phenotypic differences in cells cultured on 3D matrix. Chondrocytes in 3D system are found homogenously distributed as seen with DAPI staining (***a***). These cells start to deposit ECM component from the second week onwards (***b***) and increased proliferation is seen with phalloidin staining (***c***).

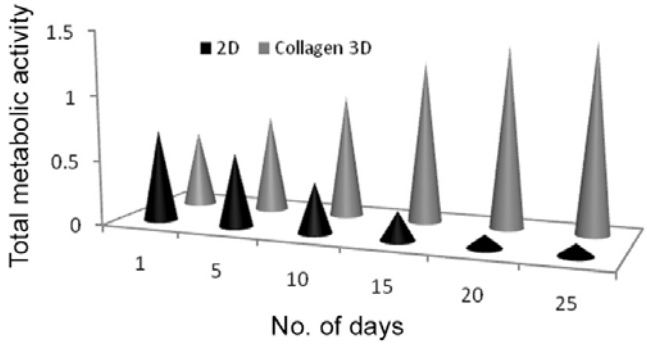

Figure 4. MTT assay performed for over 3 weeks to check for the proliferation and total metabolic activity of chondrocytes cultured on 2D and macroporous 3D system.

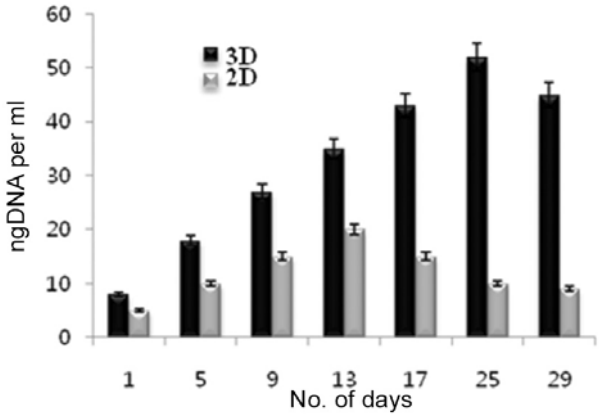

Figure 5. DNA quantification performed at regular intervals showing increase in the total DNA content of cells over four weeks. The results were found to be in accordance with MTT results.

cells in control (2D) and collagen matrix (3D) culture system was equal. However, from day 10, there was significant decline in the control, while the collagen scaffolds showed continuous increase in cellular metabolic activity with time (Figure 4). An increase in cell metabolic activity measured by MTT assay indicates good cell adhesion and proliferation in the scaffolds. Chondrocytes cannot be maintained indefinitely in culture condition once they attain confluency[8]. This is because the synthesis of sulphated-proteoglycans leads to a sharp decrease in the pH of the medium and also the lesser surface area in comparison to matrix (3D) system leads to metabolically inactive cells or cell death in control (2D) system. Much attention has been diverted towards cartilage tissue engineering and there are many studies that have utilized different approaches for culturing chondrocytes *in vitro*, like using beads or encapsulating chondrocytes[9]. However, the cells are not found to proliferate well when embedded or encapsulated inside the beads. In collagen matrix the ability of chondrocytes to retain their spherical shape confirms that the scaffold supports the cells. The cell growth rate in the collagen matrix widens its potential for tissue engineering applications. The DNA quantification results were found to be in accordance with MTT assay. Since the cells were metabolically active and proliferating, the total DNA content was also found to increase in the 3D system in comparison to 2D (Figure 5).

It is well known that GAG has an unbranched long chain and repeating disaccharide units and functions as one of the major components of ECM of the cartilage. During the chondrocytes cell culture, from day 1, constant increase in the amount of GAG was noticed in the collagen matrix (Figure 6 *a*). After day 9, the collagen matrix showed continuous increase in the production of GAG, whereas in 2D culture the GAG content was observed to decrease at every datapoint. GAG concentration was found to increase in chondrocytes cultured in clusters; and higher the cell seeding density, higher is the GAG production[10]. However, for the 3D system these cells tend to spread like fibroblasts, which affects the production of ECM components. In collagen, matrix

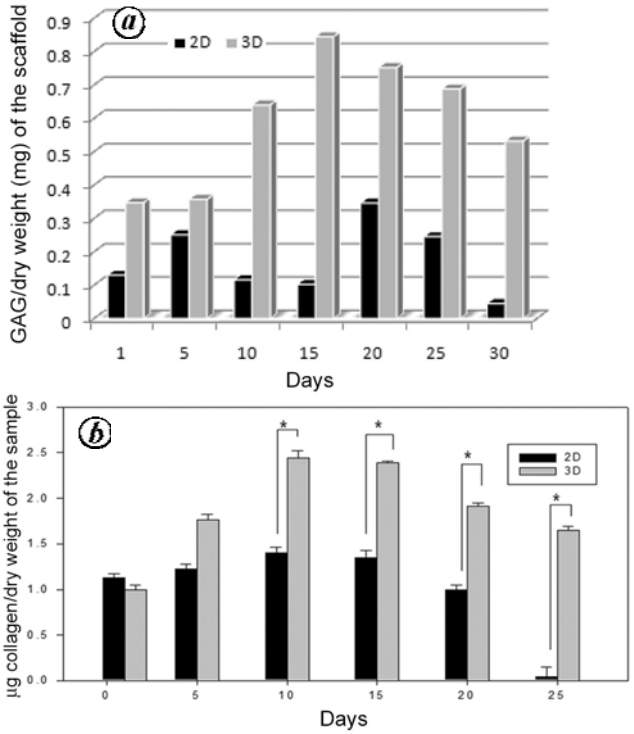

Figure 6. Biochemical analysis of GAG (***a***) and collagen (***b***) assay performed on chondrocytes cultured on collagen macroporous matrix for over weeks showing enhanced ECM component on 3D system in comparison to 2D.

chondrocytes were found to retain their original shape and showed enhanced production of GAG. The total collagen content is expressed per dry weight of the scaffold. From day 1 of cell culture in collagen scaffold, collagen content showed an upward increase and reached almost constant level from day 15 of the experiment (Figure 6 *b*). However, in 2D culture the increase in collagen content was seen up to day 9, after which there was a decline. Collagen type-I production was examined using fluorescent collagen type-I antibody that was used to cross-check the de-differentiation of primary chondrocytes towards osteoblast; it was found to be negative at every time-point. The production of GAG and collagen measured in this study indicates the cellular production of ECM, thus confirming that the scaffolds supported chondrocytes proliferation and secretion of ECM components.

In conclusion, this study demonstrated the feasibility of using collagen matrix as a scaffold for cell carrier in cartilage tissue engineering. The study presents a simple one-step processing method employed for successfully synthesizing synthetic and natural polymeric matrix scaffolds. These scaffolds show fine elastic and mechanical characteristics with controlled pore architecture, high porosity along with relatively large pore size for better cell and waste transport. The matrix does not demonstrate any failure when subjected to stress and strain procedure and the capacity to retain their shapes after drying makes these scaffold materials more effective and easy to handle. Cell–matrix interaction observed while culturing chondrocytes showed excellent cell attachment, proliferation and secretion of ECM, thus establishing the potential of matrix for tissue engineering applications, particularly cartilage tissue engineering.

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