Research Article | **Open Access**

Development of 3D Alginate Encapsulation for Better Chondrogenic Differentiation Potential than the 2D Pellet System

Tanya Debnath[1], Usha Shalini[1], Lakshmi K Kona[2], VidyaSagar JVS[3], Suguna Ratnakar Kamaraju[1], Sumanlatha Gaddam[4] and Lakshmi Kiran Chelluri[1]*

[1]Transplant Immunology and Stem Cell Laboratory, Global Hospitals, Hyderabad, India
[2]Department of Bariatric Surgery, Global Hospitals, Hyderabad, India
[3]Department of Orthopedics, Aware Global Hospitals, Hyderabad, India
[4]Department of Immunology, Bhagwan Mahavir Hospital and Research Centre, Hyderabad, India

Abstract

Introduction: Articular cartilage damage has reduced potential to regenerate. This is due to lack of blood supply at the site of injury and widely spread chondrocytes in dense extracellular matrix. Alternate strategies to regenerate the damaged tissue require exogenous supply of several chondrocyte implants. There are inherent challenges to optimize an appropriate tissue culture methodology in the enrichment of chondrocytes, thus necessitating the use of alginate hydrogel. Furthermore, human derived adipose stem cells provide an ideal tissue source to treat large focal defects.

Objectives: We explored the differentiation potential, expansion and growth kinetics of the human adipose derived stem cells (hADSCs) in 3D alginate microspheres. The growth pattern of articular chondrocytes was also studied in pellet system.

Methods: Isolated hADSCs and cartilage derived chondrocytes were cultured and characterized by flow cytometry and scanning electron microscopy (SEM). Cell viability and compatibility was studied using MTT, Annexin V FITC assays. RT-PCR was used to study chondrogenic regulation.

Results: Uniformly distributed cells encapsulated in alginate microspheres were imaged by SEM and they expressed multi-potent stem cell phenotype from the third passage. hADSCs were metabolically active within the alginate microspheres. The chondrocyte pellet culture from cartilage demonstrated lower growth potential as compared to alginate encapsulation. Apoptotic assays provided safety profile for the alginate during cell growth. The up regulation of cartilage specific genes like transforming growth factor-beta (TGF-β), collagen type-X, cartilage oligomeric matrix protein (COMP) and collagen type II was observed during the entire period of culture in alginate spheres.

Conclusion: The chondrocyte phenotype was conserved in pellet system with rich glycosaminoglycan (GAG) polysaccharides. Moreover, hADSCs could proliferate and differentiate into chondrogenic lineage within alginate matrix. Thus, an enriched chondrocyte requirement in alginate as a scaffold design would aid in the treatment of large focal defects.

Introduction

The human articular cartilage comprises of chondrocytes in dense ECM rich in glycosaminoglycans, collagen and glycoproteins. The limited ability of articular cartilage to self-repair has led to a wide variety of treatment approaches for focal chondral defects with varying levels of success. The low cell density of cartilage is also a drawback in cell transplantation as well as in chondrocyte biology. Limited proliferative and regenerative capacity of adult chondrocytes and their potential dedifferentiation upon expansion is an impediment, and hence, it is imperative to explore alternate strategies [1]. An alternative therapy for the repair of damaged articular cartilage resides in the tissue engineering approach [2]. Tissue engineering can potentially use matured cells (chondrocytes) or immature cells (mesenchymal stem cells). The adult stem cells could be the solution to differentiate into different types of cells, allowing us to regenerate damaged tissues [3].

Adipose tissue represents a good candidate tissue for obtaining adult stem cells for regenerative therapy because of least ethical implications and increased voluntary donation [4]. Furthermore, the stromal vascular portion of adipose tissue has been reported to contain up to 2% of cells that are able to differentiate into various cell types compared with only 0.002% of cells with this capability in bone marrow [5].

A wide variety of scaffolds have been used to mimic the extra

cellular matrix of the cartilage tissue *in vitro* [6]. It is known that three dimensional (3D) high cell density cultures conserves the phenotype of chondrocytes and supports the proliferation. The most common matrix material used is collagen or alginate. Sodium alginate has been widely recognized as a conventional stem cell delivery system for repair of cartilage defects and as a model for 3D culture system [7,8].

This innate feature of alginate hydrogel allows a good transfer of gases and nutrients to maintain cell viability, proliferation and differentiation. Three-dimensional systems could potentially provide greater surface to volume ratio, necessary to cope with the scale of cell expansion for allogenic applications. The induction potential of stromal cells to chondrogenic lineage was evidenced by the expression of aggrecan, collagen type II and X [9]. However, on prolonged culture and therapy these differentiated stromal cells have resulted in the development of fibro-cartilage tissue and scar formation. Failure rate of autologous chondrocyte implantation is about 60% due to de-differentiation phenomenon. It is of utmost importance that chondrocyte cell phenotype should be conserved in the development of *in vitro* chondrocyte cultures. The present study deals with the biological performance of articular chondrocytes and use of the alginate matrix with hADSCs towards chondrogenic lineage. Based on the previous reports, calcium chloride was selected as cross linker due to its chelating properties as compared to others. The study parameters in 3D alginate matrices as temporary physical support for hADSCs included, the cell proliferation, viability, compatibility and chondrocyte gene expression during differentiation. Thus, the current study would provide a preliminary insight to design a strategy for encapsulation and differentiation of hADSCs within the alginates. This may pave the way for further studies in regenerating the injured tissues.

Materials and Methods

Dulbecco's Modified Eagle Medium (Low glucose -DMEM; 4.5 mmol/L glucose), type I collagenase, and fetal bovine serum (FBS), sodium alginate were purchased from Gibco (BRL, USA). All antibodies were purchased from BD Bioscience (San Jose, USA). Chemicals were purchased from Sigma-Aldrich (St. Louis, USA). Prior written informed consent from the patients was obtained after taking clearance from the Global Hospitals, Institutional Ethical (IEC) with Ref no. GMERF/BS/SAC/IEC/IC_SCR2014/01R3.The samples for the study were collected in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki).Human adipose tissue was collected from morbid obese patients of both genders within 20-45 years age group undergoing bariatric surgery. The samples were collected in L-DMEM and pooled for further experimentation to circumvent the age variability. The cartilage scrapes were collected in sterile L-DMEM medium supplemented with antibiotics during arthroscopy procedure. Gender was not biased for the study and samples were collected from patients within 20-45 years age group.

Isolation of chondrocytes from cartilage tissue

The cartilage scrapes of 2×2 mm bits were subjected to low speed magnetic stirring at 37°C for 45 min in 0.25% trypsin. The cells dispensed in the media are re-suspended in 0.2% collagenase solution in a CO_2 incubator at 37°C for 90 min. The digested tissue was filtered using 70 μm cell strainer to separate the chondrocytes from undigested ECM. The cells were cultured as pellet in chondrocyte growth media containing L-DMEM supplemented with 10% FBS, 1% ITS-premix in the presence of 10 ng/ml human TGF-β2 and at a density of 5×10^4 cells/ml in a humid, 5% CO_2 incubator at 37°C. The pellet culture was maintained in a centrifuge tube for 21 days, with the medium replaced at 3 day interval. The sedimented cells formed spherical aggregates at the bottom of the tube.

Morphological and histological analysis of pellet

Pellets were harvested after 21 days, fixed in 10% buffered formalin for 2 h, and kept in 70% ethanol overnight. Samples were embedded in paraffin and 5 μm sections were cut. Hematoxylin and Eosin (H&E) staining of paraffin sections was done for evaluation of cell morphology in pellets. Sulfated GAGs were visualized by staining with 0.5% alcian blue for 10 min.

Cell growth kinetics of chondrocytes

The growth rate of human chondrocytes was measured by seeding initial cell density at 0.4×10^5 cells/ml. The cells were periodically counted to check their growth profile (n=5).

Isolation and characterization of human adipose derived stem cells

Tissue fragments were washed intensely with phosphate buffer saline (PBS) minced properly and digested with collagenase type I (1 mg/ml) for 30–60 min in a humidified atmosphere of 95% air and 5% CO_2 at 37°C with gentle agitation. The digested tissue was filtered through a 40 μm cell strainer (BD Biosciences, USA) and cells were centrifuged at 1,800 rpm for 5 min. The cell pellet was re-suspended in L-DMEM supplemented with 10% FBS, penicillin (100 IU/ml), streptomycin (100 IU/ml), gentamycin (50 IU/ml), amphotericin B (2.5 μg/ml) and b-FGF (10 ng/ml) and plated at a density of $5 \times 10^4/cm^2$ in polystyrene T25 culture flasks and were incubated in a humid 5% CO_2 incubator at 37°C for 48 h. Non adherent cells were discarded and adherent cells were cultured in complete culture media by replenishing fresh media at 3 days interval. The hADSCs were harvested at 80% confluence and passaged further. Cell surface antigen phenotype by flow cytometry was performed at each passage to characterize the hADSCs. Cells were stained with CD90-FITC, CD73-APC antibodies (positive markers) and CD34-PE, CD45-FITC, HLA-DR–PE antibodies (negative marker) to establish the expression of the multipotent stem cells using Cell Quest Software (Becton Dickinson).

Preparation and encapsulation of hADSCs in sodium alginate beads

The hADSCs cell suspension was adjusted to 2×10^6 cells/ml and added to an equal volume of 4% (w/v) alginate to give a final concentration of 1×10^6 cell/ml in 2% (w/v) alginate. The cell/alginate suspension was slowly dropped through a 23-gauge needle into 100 mM $CaCl_2$ solution in PBS. The resultant beads were incubated in the $CaCl_2$ for 10 min at room temperature to induce cross linking of the alginate. The beads were subsequently washed in 4 changes of L-DMEM and 20% FBS.

Characterization of hADSCs incorporated in alginate Systems

Scanning electron microscopy (SEM) analysis: The encapsulated hADSCs within sodium alginate, maintained in culture for 7 days, were washed twice with PBS and fixed for 6 h at 4°C with 2.5% glutaraldehyde (Sigma-Aldrich, Co) in PBS, containing 0.1% $CaCl_2$. After 2 h, the samples were dehydrated in a series of graded alcohols and were dried to a critical point using an electron microscopy science critical point drying (CPD) unit. Samples were then scanned at various magnifications using a scanning electron microscope (Model: JOEL-JSM 5600, JAPAN) of the Department of Veterinary Pathology, Ruska Laboratories, (Hyderabad, India).

Assessment of cell viability and metabolic activity: Disruption of mitochondria is observed in early stages of cell death and JC-1 dye (Molecular Probes, Invitrogen detection technologies, Eugene, Oregon) is widely used to monitor mitochondrial health. JC-1 (5,5,6,6'-tetrachloro-1,1'3,3'tetraethylbenzimidazole carbocyanine iodide)forms aggregates in mitochondria created due to potential difference and thus, discriminating the healthy cells with intact mitochondria against the dead cells. The alginate encapsulated cells were stained with 2 µg/ml of JC-1 and incubated for 30 min. Images were taken in red and green filters using confocal microscope (Leica, TCS SPE).

The cell metabolism within the alginate bead was assessed by MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) assay by the standard protocol of Mossman et al. [10]. The samples were run in triplicates wherein alginate beads without cells were taken as control (n=6).

Apoptosis Assay: Cell death was determined using Annexin-V-FITC/propidium iodide assay to distinguish the necrotic and apoptotic cells. Cells that stain positive for Annexin-V-FITC and negative for propidium iodide would undergo apoptosis, and those which are negative for both Annexin-V-FITC and PI are deemed to be alive. Cells were revived from the beads using dissolution buffer which constitutes of 55 mM EDTA, 10 mM HEPES, maintained at pH 7.4 and were stained with Annexin-V FITC as per manufacturer's recommendations (eBiosciences, USA). The assay was performed three times for reproducibility.

Chondrogenic differentiation of encapsulated hADSCs in alginate beads

To induce chondrogenic differentiation, encapsulated hADSCs were cultured in high-glucose DMEM supplemented with 10% FBS, 50 mM ascorbic acid-2-phosphate, 0.1 µM dexamethasone, 1 mM sodium pyruvate and 10 ng/ml TGF-β2 for 21 days. The culture media was changed every 3 days. After defined period the beads were washed with PBS and dissolved with dissolution buffer for 15 min. The cells were centrifuged and the resultant pellet was stored for further use.

Morphological evaluation of induced encapsulated cells: After 21 days of hADSCs culture in the differentiation media, the alginate micro carriers were fixed in 4% paraformaldehyde for 15 min, later differentiated cells were stained with hematoxylin and eosin for morphological evaluation. Sections (10 µm) were stained with 1% Alcian blue and Masson's trichrome, and were counterstained with acid fast red (Sigma-Aldrich).

qPCR for chondrocyte specific gene: Relative real-time PCR was performed to assess exact expression ratio of a set of chondrocyte genes and the rate of their regulatory mechanisms in chondrocytes during different time points cultured in alginate (n=3). Total RNA was prepared from RNeasy mini kit (QIAGEN, Germany). Standard RT reactions were performed with 2 mg total RNA using Random hexamer as a primer and a cDNA was synthesized. Real-time PCR was carried

out on the StepOne™ Real Time PCR system, using the SyGr master mix (Applied Biosystems, Carlsbad, CA, USA) at final volume of 20 µl using standard PCR conditions (40 cycles with an annealing/elongation step at 60°C). The relative expression of each gene was normalized against β2M, the house-keeping gene. Table 1 depicts the primer sequences for chondrocyte genes (OriGene technologies, USA). The relative expression of each target gene was analyzed by $2^{-\Delta\Delta Ct}$ method and the gene expression was calculated as a fold increase periodically.

Statistical analysis: SPSS package was used for all the statistical analysis. Mean values ± SD (standard deviation) were calculated for data obtained from the assays for proliferation as well as that obtained from real-time PCR. Significant values at $p<0.05$ were calculated using Student's 't' test.

Results

Chondrocytes culture and characterization

The sedimentation of the isolated chondrocyte pellet into single aggregate was observed after 24 h. During cultivation, the size of the pellet continuously increased, with high cellular density and the pellets became opaque. The rounded morphology was evident by analyzing sections of pellet cultures stained for H&E and alcian blue. Hematoxylin and Eosin (H&E) sections showed a homogenous cell distribution in the pellet with isogenous cell groups. The center region contained rounded cells with darkly stained nuclei and surrounded by extracellular matrix, resembling the pericellular matrix of the chondrocytes. Alcian blue staining showed deposits of acid mucopolysaccharides in the proximity of the cells which is the deterministic feature of the sulfated glycosaminoglycans by cells cultured in the pellet culture (Figure 1a).

The growth kinetics of the human chondrocytes was evaluated by plotting the growth curve (n=5). It was observed that the culture underwent an initial lag phase without increase in cell number for 7 days. The cell multiplication started exhibiting the log phase with rapid increase in cell number upto 28 days and later the cell number declined to 7×10^4 cells/ml over 10 days (Figure 1b).
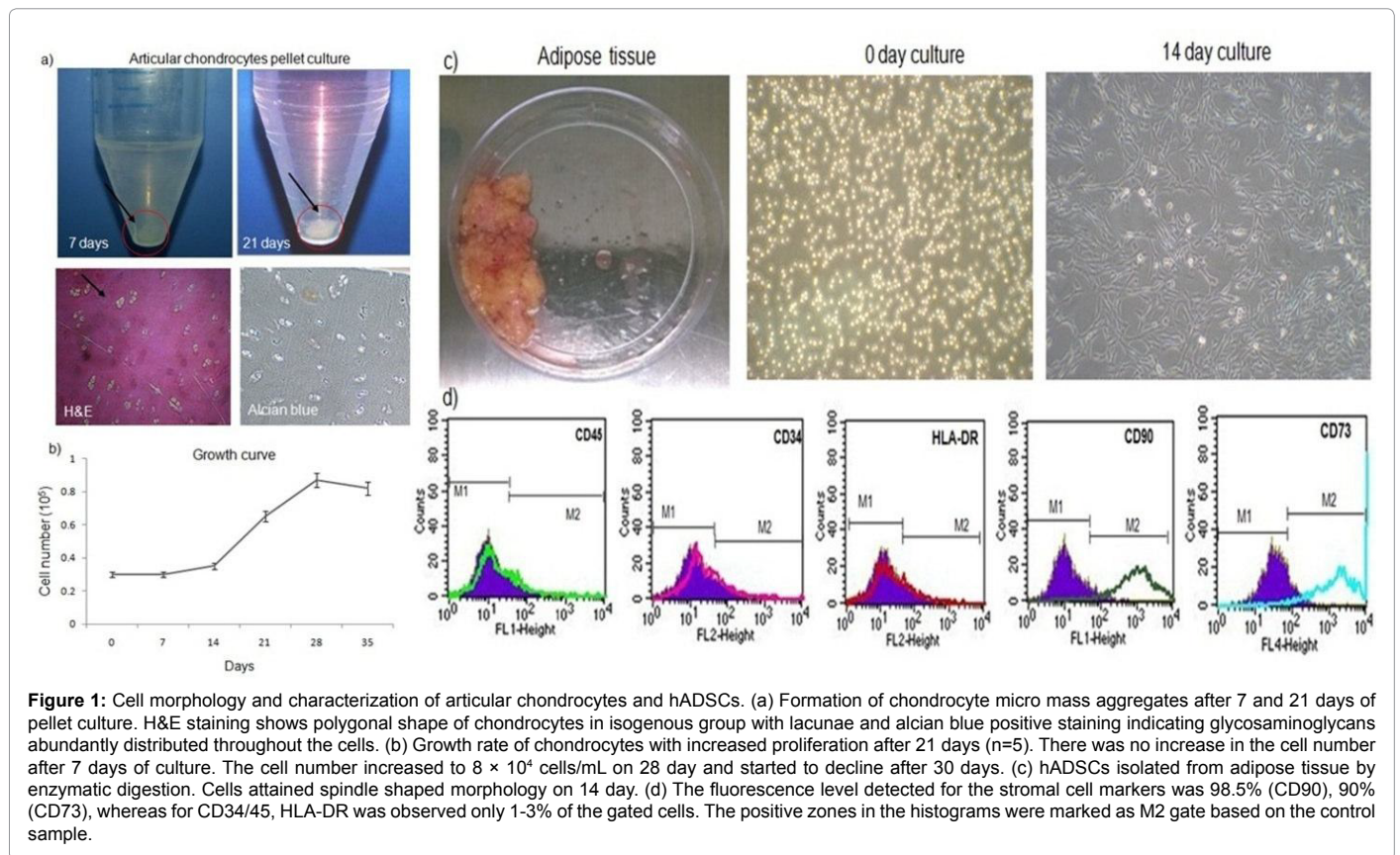
Isolation and characterization of mesenchymal stem cells from adipose tissue: An average yield of 1×10^6 cells/gm was obtained from 2 gm of adipose tissue. As the cells were propagated in monolayer culture, they showed a more uniform fibroblast-like morphology. hADSCs demonstrated spindle shape and fibroblast-like appearance on 14th day of culture (Figure 1c). The third passage hADSCs exhibited a distinct phenotype of MSC origin. Based on these results, we established that the most suitable hADSCs for incorporation into the alginate matrix were the cells corresponding to the third cell passage.

In vitro behavior of hADSCs embedded into alginate hydrogels

Evaluation of the cell-laden alginate beads: The morphology of the cells encapsulated in alginate hydrogels was round in shape, identical in size as shown by giemsa staining. The hADSCs were discretely scattered

Gene	Forward primer	Reverse primer
COMP	5'-GGAGATGCTTGTGACAGCGATC-3'	5'-TGAGTCCTCCTGGGCACTGTTA-3'
CCR1	5'-CAACTCCGTGCCAGAAGGTGAA-3'	5'-GTTCAGGAGGTAGATGCTGGTC-3'
Collagen type X	5'-GGTGACACTGGTTTACCTGGCT-3'	5'-GCCTTTCCAGAGGAATGTCCTC-3'
Collagen type II	5'-CCTGGCAAAGATGGTGAGACAG-3'	5'-CCTGGTTTTCCACCTTCACCTG-3'
SOX9	5'-AGGAAGCTCGCGGACCAGTAC-3'	5'-GGTGGTCCTTCTTGTGCTGCAC-3'
TGF-β	5'-TACCTGAACCCGTGTTGCTCTC-3'	5'-GTTGCTGAGGTATCGCCAGGAA-3'

Table 1: List of cartilage specific primer sequences with 5'-3' forward and reverse oligonucleotide used for qPCR.

Figure 1: Cell morphology and characterization of articular chondrocytes and hADSCs. (a) Formation of chondrocyte micro mass aggregates after 7 and 21 days of pellet culture. H&E staining shows polygonal shape of chondrocytes in isogenous group with lacunae and alcian blue positive staining indicating glycosaminoglycans abundantly distributed throughout the cells. (b) Growth rate of chondrocytes with increased proliferation after 21 days (n=5). There was no increase in the cell number after 7 days of culture. The cell number increased to 8×10^4 cells/mL on 28 day and started to decline after 30 days. (c) hADSCs isolated from adipose tissue by enzymatic digestion. Cells attained spindle shaped morphology on 14 day. (d) The fluorescence level detected for the stromal cell markers was 98.5% (CD90), 90% (CD73), whereas for CD34/45, HLA-DR was observed only 1-3% of the gated cells. The positive zones in the histograms were marked as M2 gate based on the control sample.

all through the bead, and there were no differences in shape and density from the peripheral to the center of the bead (Figure 2a).

The investigation of the construct surface using SEM after 7 days of cultivation revealed the presence of numerous cells in the alginate matrix. SEM images of relevant cross-sections showed that hADSCs were distributed in the matrix and exhibited round-shaped morphology (Figure 2b).

hADSCs viability and proliferation within the alginate bead: The viability of alginate encapsulated hADSCs was evaluated, using a JC-1 staining. Most of the hADSCs were observed viable as evidenced by the intact mitochondrial membrane potential in fluorescent staining assay (Figure 2c).

The metabolic activity of mesenchymal stem cells grown on the hydrogel matrix was confirmed using MTT assay over a period of 7 d. (n=6). The cell seeding density of 3×10^3 cells/well provided sufficient cell-cell contact. The relative cell viability of hADSCs on the hydrogel increased >90% after 4 days. A high number of growing cells converting MTT to formazan crystals were noticed in the cells encapsulated in alginate matrix (Figure 2d).

Annexin V assay: Cells cultured within alginate beads were analyzed for phosphatidylserine (PS) exposure (Annexin-V labeling) by flow cytometry. Cells were found viable after 21 days of culture. Flow cytometry analysis was performed using FL1-FITC fluorescence histogram. The staining revealed <20% cells were apoptotic within alginate encapsulation indicating non-toxic effect. The histogram shows the presence of 66% apoptosis in the induced cells with 20 μM H_2O_2 (Figure 2e). Similar observations were made in all the three samples.

Chondrogenic differentiation of hADSCs in the alginate bead system

Encapsulated hADSCs were subjected to chondrogenic medium supplemented with TGF-β2 for 4 weeks. The differentiated cells showed deposition of a mucopolysaccharide rich extracellular matrix, as determined histologically by alcian blue staining. The masson's trichrome staining demonstrated the presence of collagen in the differentiated cell population (Figure 3a).

In order to demonstrate the differentiation potential of hADSCs towards chondrogenic lineage within alginate capsules, cells were cultured in defined chondrogenic medium supplemented with TGF-β2. To characterize the kinetics of the changes in gene expression occurring as a consequence of chondrogenic differentiation within alginate matrices, we performed quantitative RT-PCR of a number of well-known chondrogenic marker genes namely, collagen types , II and X,COMP,TGF-β, SOX9 and CCR1 in the cultured cells (n=3). qPCR analysis distinctly has shown confirming to the translational signals during the chondrogenesis differentiation pathway. CCR1, a chemokine receptor is also known to demonstrate the differentiation signals of stem cells to a chondrocyte.CCR1 were up regulated after 14 days culture. COMP and SOX9 were expressed from initial days in the culture and showed an up regulation on day 14. TGF-β expression continued to increase during the culture and exhibited up to two folds higher expression, on 21 days of culture. The major chondrogenic markers collagen type II, X demonstrated approximately three fold increase in their expression over 21 days of culture. The cartilage-specific genes have shown a progressive increase in their expression heading towards the matured chondrocyte lineage during differentiation hADSCs in 3D alginate bead (Figure 3b).
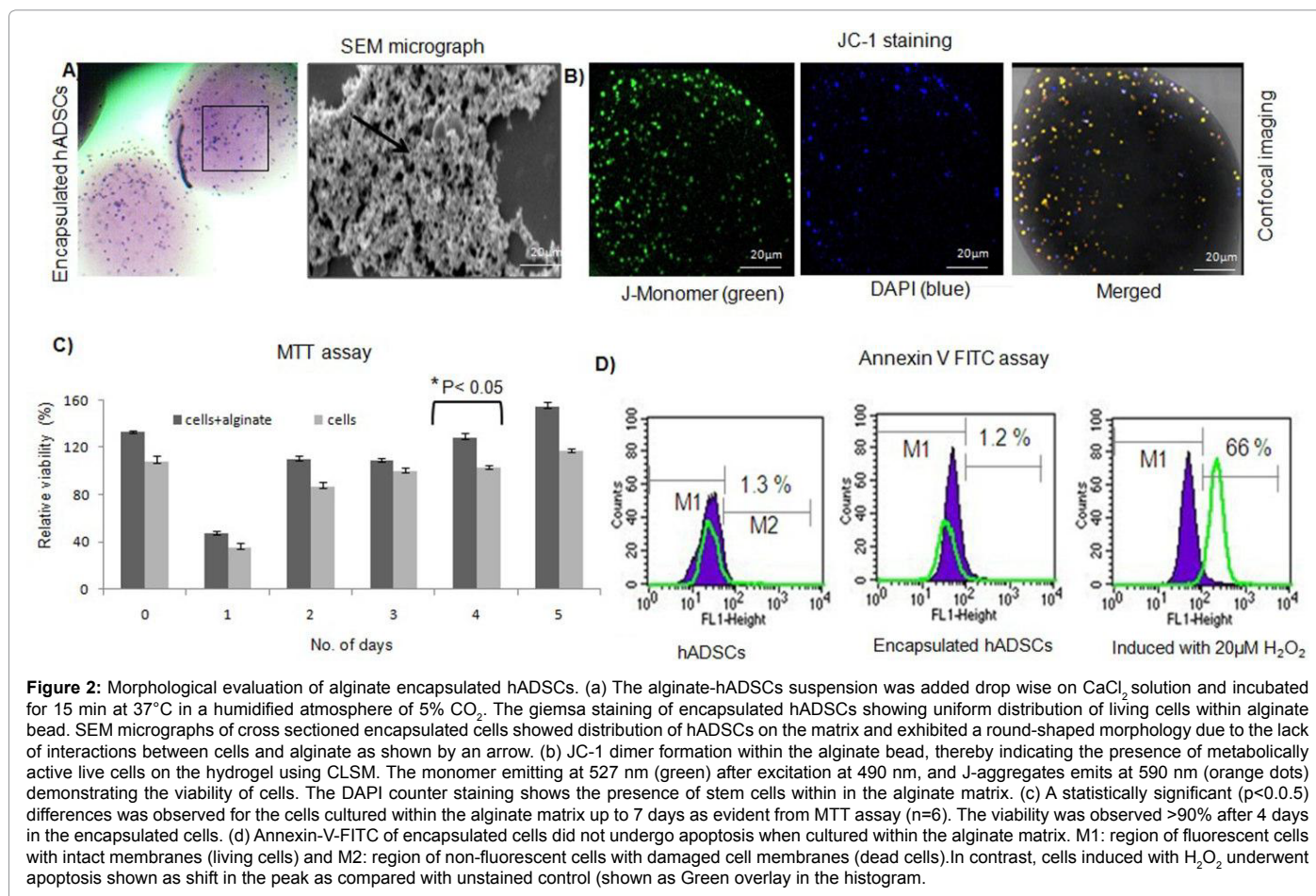
Figure 2: Morphological evaluation of alginate encapsulated hADSCs. (a) The alginate-hADSCs suspension was added drop wise on $CaCl_2$ solution and incubated for 15 min at 37°C in a humidified atmosphere of 5% CO_2. The giemsa staining of encapsulated hADSCs showing uniform distribution of living cells within alginate bead. SEM micrographs of cross sectioned encapsulated cells showed distribution of hADSCs on the matrix and exhibited a round-shaped morphology due to the lack of interactions between cells and alginate as shown by an arrow. (b) JC-1 dimer formation within the alginate bead, thereby indicating the presence of metabolically active live cells on the hydrogel using CLSM. The monomer emitting at 527 nm (green) after excitation at 490 nm, and J-aggregates emits at 590 nm (orange dots) demonstrating the viability of cells. The DAPI counter staining shows the presence of stem cells within in the alginate matrix. (c) A statistically significant (p<0.0.5) differences was observed for the cells cultured within the alginate matrix up to 7 days as evident from MTT assay (n=6). The viability was observed >90% after 4 days in the encapsulated cells. (d) Annexin-V-FITC of encapsulated cells did not undergo apoptosis when cultured within the alginate matrix. M1: region of fluorescent cells with intact membranes (living cells) and M2: region of non-fluorescent cells with damaged cell membranes (dead cells).In contrast, cells induced with H_2O_2 underwent apoptosis shown as shift in the peak as compared with unstained control (shown as Green overlay in the histogram.

The expression of chondrocyte specific genes was studied using a grid based system to assess the levels of gene expression on different time points. The genes expression of alginate encapsulated cells was analyzed parallel with time of differentiation thus understanding the matrix and stem cells interactions (Figure 3c).

Discussion

In the present study we cultured articular cartilage derived chondrocytes in the pellet culture system. Chondrocytes require 3-D environment in order to conserve their phenotype. In monolayer culture these cells dedifferentiate and produce matrix components characteristic of fibro-cartilage. This result in variable gene expression pattern [11]. The growth profile of the chondrocytes indicates restricted proliferation potential with only limited number of cells, making it less feasible for clinical transplantation in case of injuries.

Tissue engineering deals with the use of growth factors for stem cell differentiation into specific lineage. But currently the research is directed towards the use of biomaterials as scaffolds for cell differentiation and regeneration.3D culture systems have frequently been used to mimic *in vivo* conditions [12,13]. Our previous reports have confirmed that hADSCs can be easily harvested, cultured and differentiated into chondrogenic lineage [14]. Based on their morphological features and phenotypic analysis by flow-cytometry, cells from the third passage were used in the 3-D culture systems. We analyzed the *in vitro* behavior of hADSCs within this matrix. The hADSCs were encapsulated in alginate matrix, and their morphology, proliferation capacity and chondrogenic

differentiation were continuously monitored. Additionally, the cell viability remained high throughout the period, a finding consistent with our previous observations with PLL-HA encapsulation [15]. Earlier studies have revealed that most of the encapsulated stem cells are retained in their G0–G1 phase whereas less number of cells in S phase. This suggests coordinated cell proliferation in an appropriate 3D state [16]. Thus, the 3D encapsulation technique will direct definite lineage differentiation of hADSCs instead of propagation. Examination of micrographs confirmed these findings, with spherical cell morphology. MTT assays provided evidence that alginate hydrogels stimulated cell proliferation, as demonstrated by the increased number of hADSCs within alginate, with prolonged incubation period. The alginates do not induce cytotoxicity as evident from mitochondrial staining by JC-1. Cells expanded within alginate typically exhibited a greater viability as shown by Annexin V assay. There was no significant apoptosis after two weeks of culture indicating higher metabolism and non-toxicity of alginate on the cultured cells. The cell viability data is also consistent with a previous finding where the cells remained viable throughout the culture duration. These results are in consonance with the observation that the alginate matrices provides a structure formed by interconnected pores suitable to accommodate the hADSCs, viability, nutrient and protein transport [17].

It is well known that the TGF-β class of growth factors is able to induce chondrocyte differentiation in mesenchymal stem cells. The present study is based on the use of the TGF-β2 supplemented media in culture conditions to induce serial expression of cartilage specific
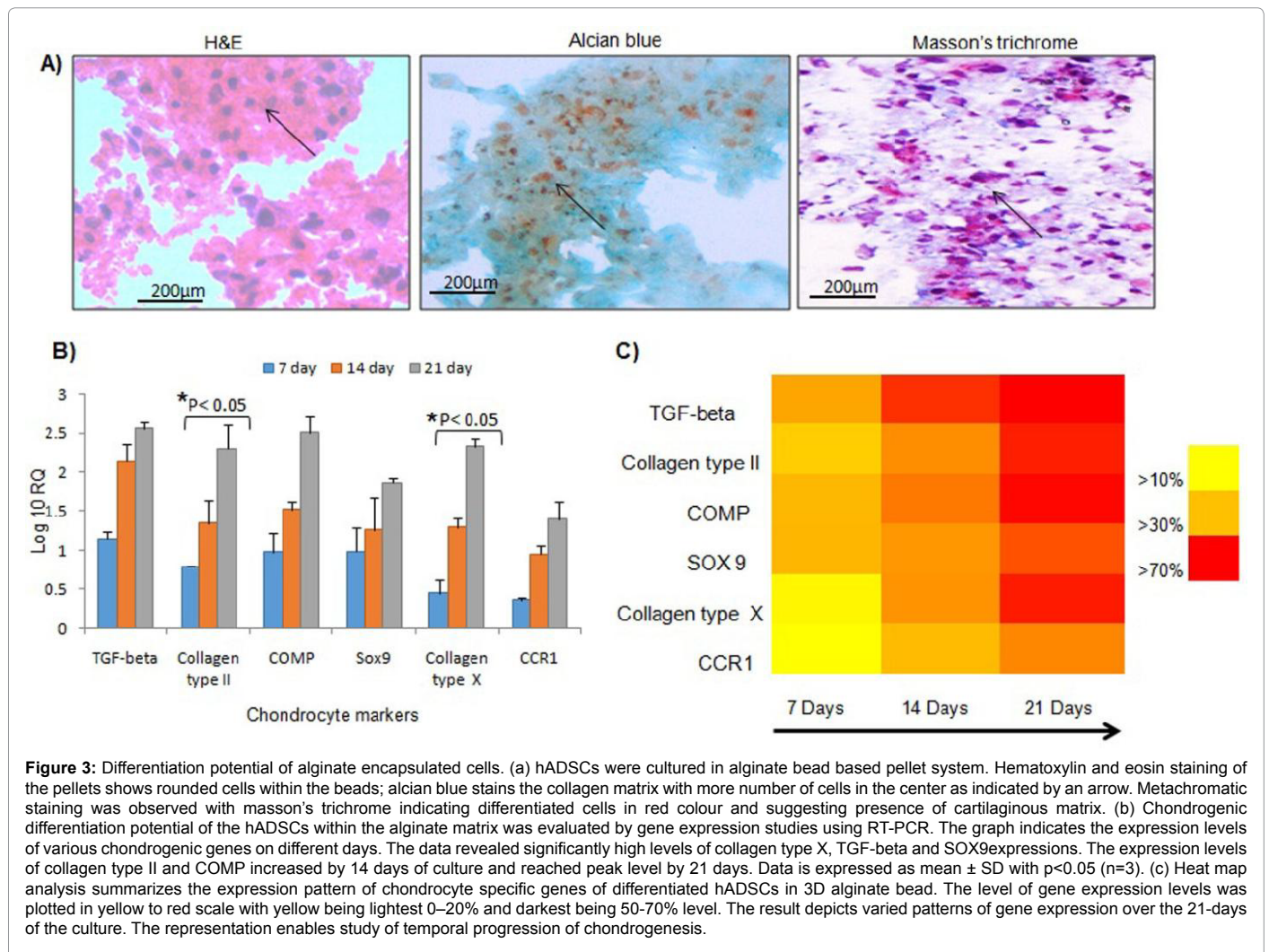
Figure 3: Differentiation potential of alginate encapsulated cells. (a) hADSCs were cultured in alginate bead based pellet system. Hematoxylin and eosin staining of the pellets shows rounded cells within the beads; alcian blue stains the collagen matrix with more number of cells in the center as indicated by an arrow. Metachromatic staining was observed with masson's trichrome indicating differentiated cells in red colour and suggesting presence of cartilaginous matrix. (b) Chondrogenic differentiation potential of the hADSCs within the alginate matrix was evaluated by gene expression studies using RT-PCR. The graph indicates the expression levels of various chondrogenic genes on different days. The data revealed significantly high levels of collagen type X, TGF-beta and SOX9expressions. The expression levels of collagen type II and COMP increased by 14 days of culture and reached peak level by 21 days. Data is expressed as mean ± SD with p<0.05 (n=3). (c) Heat map analysis summarizes the expression pattern of chondrocyte specific genes of differentiated hADSCs in 3D alginate bead. The level of gene expression levels was plotted in yellow to red scale with yellow being lightest 0–20% and darkest being 50-70% level. The result depicts varied patterns of gene expression over the 21-days of the culture. The representation enables study of temporal progression of chondrogenesis.

genes in an alginate encapsulated culture system. According to Hani et al., the cell entrapment imposes a spherical cell morphology which has beneficial effects towards chondrogenesis along increased proliferation [18].

The gene expression study also illustrated a progressive increase of chondrocyte progenitors than matured chondrocytes and finally the expression of hypertrophic genes. Further, the observations strengthened the presence of collagen type X, collagen type II, SOX9 and COMP expression in the cells within beads. Their expression reached peak levels at days 10 to 14, and these were maintained at high expression levels at later stages. Substantially high expression of collagen type II and X in the alginate bead culture system is consistent with earlier studies on the expression pattern of these genes during chondrocytic differentiation *in vitro* [19]. As reported in other relevant studies, up-regulation of collagen type X, COMP appeared to be advanced, relative to patterns that have been historically reported *in vivo* [20]. Chondrocyte gene expression mechanism is also influenced by various transcriptional and translational factors that are involved in cell-matrix interactions. The series of chondrocyte specific gene expression of hADSCs undergoing chondrogenesis within the 3D alginate bead was well demonstrated by RT-PCR. The heat map data also demonstrated the stage specific expression for chondrocytic differentiation. The upregulation of cartilage specific markers like

collagen type II and X, COMP was observed as similar to our qPCR results after 21 days of culture. Thus, the increased level of collagen type II expression suggests that the cells within the alginate bead could differentiate and became hypertrophic.

The chondrocyte culture system represents very low ratios of surface to volume, making them inefficient in terms of scalability. The number of culture units has to be remarkably increased to get significantly increased articular chondrocytes. The process is time consuming and laborious. However, alginate microenvironments provide support to hADSCs to increase in number and remain viable. These matrices do not alter the cell morphology and create conditions that are favorable for chondrogenic differentiation. An enhancement of genotypic expression patterns similar to that of chondrogenic lineage was observed in the case of alginate micro-carrier. It is suggested that alginate based matrices have promising application in cell delivery for soft tissue repair and regeneration.

References

1. Hunziker EB (2002) Articular cartilage repair: basic science and clinical progress A review of the current status and prospects. Osteoarthr Cartilage 10: 432-463. [PubMed]

2. Zhang L, Hu J, Athanasiou KA (2009) The role of tissue engineering in articular cartilage repair and regeneration. Crit Rev Biomed Eng 37: 1-57. [PubMed]

3. Guilak F, Awad HA, Fermor B, Leddy HA, Gimble JM (2004) Adipose-derived adult stem cells for cartilage tissue engineering. Biorheology 41: 389-399. [PubMed]

4. Patrick CW, Zheng B, Johnston C, Reece GP (2002) Long-term implantation of pre-adipocyte-seeded PLGA scaffolds. Tissue Eng 8: 283-293. [PubMed]

5. De Ugarte DA, Morizono K, Elbarbary A, Alfonso ZC, Zuk PA, et al. (2003) Comparison of multi-lineage cells from human adipose tissue and bone marrow. Cells Tissues Organs 174: 101-109. [PubMed]

6. Brahatheeswaran D, Yasuhiko Y, Toru M, Sakthi KD (2011) Polymeric scaffolds in tissue engineering application: A Review. Int J Polym Sci.

7. Diekman BO, Rowland CR, Lennon DP, Caplan AI, Guilak F (2010) Chondrogenesis of adult stem cells from adipose tissue and bone marrow: induction by growth factors and cartilage-derived matrix. Tissue Eng Part A 16: 523-533. [PubMed]

8. Neuss S, Apel C, Buttler P, Denecke B, Dhanasingh A, et al. (2008) Assessment of stem cell/biomaterial combinations forstem cell-based tissue engineering. Biomaterials 29: 302-313. [PubMed]

9. Masuda K, Sah RL, Hejna MJ, Thonar EJ (2003) A novel two-step method for the formation of tissue-engineered cartilage by mature bovine chondrocytes: the alginate-recovered-chondrocyte (ARC) method. J Orthop Res 21: 139-148. [PubMed]

10. Mossman T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55-63. [PubMed]

11. Girandon L, Kregar-Velikonja N, Bozikov K, Barlik A (2011) In vitro models for adipose tissue engineering with adipose-derived stem cells using different scaffolds of natural origin. Folia Biol 57: 47-56. [PubMed]

12. Payne KF, Balasundaram I, Deb S, Di Silvio L, Fan KF (2014) Tissue engineering technology and its possible applications in oral and maxillofacial surgery. Br J Oral Maxillofac Surg 52: 7-15. [PubMed]

13. Goessler UR, Hormann K, Riedel F (2004) Tissue engineering with chondrocyte and function of the extracellular matrix (Review). Intern J Mol Med 13: 505-513. [PubMed]

14. Debnath T, Ghosh S, Potlapuvu US, Kona L, Kamaraju SR, et al. (2015) Proliferation and differentiation potential of human adipose-derived stem cells grown on chitosan hydrogel. PLoS One 10: e0120803. [PubMed]

15. Parul G, Tanya D, Lakshmi KC, Neha H (2012) Feasibility of polymer based cell Encapsulation using electrostatic layer by layer assembly. J Biomater Tissue Eng 2: 215-219.

16. Waqar H, Yixiao D, Wenxin W (2013) Encapsulation and 3D culture of human adipose-derived stem cells in an in-situ crosslinked hybrid hydrogel composed of PEG-based hyper branched copolymer and hyaluronic acid. Stem Cell Res Ther 4: 32. [PubMed]

17. Lakshmi KC (2012) Stem cells and extracellular matrices. Morgan and Claypool Life Sciences.

18. Awad HA, Wickham MQ, Leddy HA, Gimble JM, Guilak F (2004) Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. Biomaterials 25: 3211-3222. [PubMed]

19. Khosravizadeh Z, Razavi S, Bahramian H, Kazemi M (2014) The beneficial effect of encapsulated human adipose-derived stem cells in alginate hydrogel on neural differentiation. J Biomed Mater Res B 102: 749-755. [PubMed]

20. Xu J, Wang W, Ludeman M, Cheng K, Hayami T, et al. (2008) Chondrogenic differentiation of human mesenchymal stem cells in three-dimensional alginate gels. Tissue Eng Part A 14: 667-680. [PubMed]