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In vitro evaluation of alginate encapsulated adipose-tissue stromal cells for use as injectable bone graft substitute

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Abstract

This study aims to investigate the survival and osteogenic behavior of murine-derived adipose-tissue stromal cells (ATSCs) encapsulated in alginate microcapsules thereby instigating further studies in this cell delivery strategy for in vivo osteogenesis. Cell viability was quantified using a tetrazolium-based assay and osteogenic differentiation was evaluated by both alkaline-phosphatase (ALP) histochemistry and osteocalcin mRNA analysis. Following microencapsulation, cell numbers increased from 3.9×10^3 on day 1 to 7.8×10^3 on day 7 and maintained excellent viability in the course of 21-day culture. ALP was 6.9, 5.5, and 3.2 times higher than monolayer cultures on days 7, 14, and 21, respectively. In addition, osteocalcin mRNA was detectable in encapsulated cultures earlier (day 14) than monolayer cultures. We conclude that alginate microcapsules can act as three-dimensional matrix for ATSC proliferation and has potential for use as injectable, biodegradable scaffold in bone tissue engineering.

Keywords: Ca–alginate; Microcapsules; Cell transplantation; Adipose-tissue stromal cells; Bone tissue engineering

Multipotent adult progenitor cells that can differentiate into a variety of specific somatic cell types for tissue engineering purposes have been isolated from the stromal compartment of many tissues including bone marrow [1], periosteom [2], synovial membrane [3], and subcutaneous fat [4,5]. Several recent reports have indicated that cells from the stromal-vascular fraction of adipose tissue possess phenotypic and functional characteristics similar to mesenchymal stem cells (MSC) derived from the bone marrow. These cells have been called, adipose tissue stromal cells (ATSC), and are known to be pluripotent, easily harvestable with lower donor site morbidity when compared to other pluripotent stem cell sources [4]. In addition, ATSCs attached and proliferate easily in culture and are, therefore, available on large scale even for autologous grafting in small animals like rodents. Furthermore, ATSCs are reported to possess differentiation potentials comparable to bone marrow stromal cells when cultured on 2-D monolayer plastic surfaces [4] and have been proposed for use in combination with 3D scaffolds as bone graft substitute in vivo.

Several injectable 3D scaffolds have been used as bone graft substitutes [6,7]. However, none has gained universal acceptance. As an important biotechnology in tissue engineering, cell encapsulation has increasingly attracted interests in recent years [8,9]. It promotes tissue regeneration by facilitating the localized retention of viable cells [10,11], thereby permitting the release of therapeutic agents to the host over a desirable period of time [12].

The ideal characteristics of microcapsules should be suitable size for ease of injection, biocompatibility with the resident cells and surrounding tissues for ease of

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integration, and biodegradability to eliminate the necessity of secondary surgery for removal of materials.

Both natural materials such as collagen, fibrin, alginate, chitosan, and synthetic polymers including those based on acrylamides, 2 poly(vinyl alcohol) (PVA), 3 poly(ethylene glycol) (PEG), and 4–6 (poly(ethylene glycol) fumarate) (OPF) have been developed for this application [13]. Among all the natural and synthetic materials, polyanion alginate possesses excellent biocompatibility and in vivo stability [14–16]. In this study, the effect of confinement within calcium cross-linked alginate microcapsules on the survival of cells with osteogenic potentials and their subsequent ability to elicit osteogenic response was investigated in vitro. This study was performed to investigate whether or not alginate microcapsules confining ATSC are potentially injectable materials for new bone formation.

Materials and methods

Intermediate guluronic (G) sodium alginate obtained from Sigma was purified according to the method proposed by de Vos et al. [16]. Dulbecco's modified Eagle's medium (DMEM), penicillin/streptomycin, Trypsin/EDTA, and L-glutamine were purchased from GIBCO (NY, USA). Fetal calf serum (FCS) was from Biocell Laboratories (CA, USA). Dexamethasone (Dex), ascorbate-2-phosphate, sodium β-glycerophosphate (β-GP), collagenase type I, Alkaline phosphatase Diagnostic Kit-104LS, and Sigma Fast Red were from Sigma (TX, USA). Bradford protein assay kit 1 was from Bio-Rad, Hercules, CA, and cell-culture wares and disposables were from Corning®.

Fabrication of alginate microcapsules. Alginate microcapsules were produced by the air-jet technique, using a semi-automatic droplet generator assembled in our laboratory based on principles described by Sun et al. [12] and details reported previously [17]. From pilot studies, the most suitable parameters of the droplet generator were determined and summarized in Table 1.

To prepare microcapsules, purified alginate was dissolved at 4 °C in Krebs–Ringer–Hepes (1.5% w/v) and filtered through 0.22 μm filter (mixed cellulose ester membrane). The alginate/ATSC mixture droplets were gelled in a 200 mM $CaCl_2$ solution for 5 min to form solid core, microcapsules.

Characterization of microcapsules. The shape and size of microcapsules as well as the average number of cells in a microcapsule were evaluated with a bright field inverted microscope (Leica DM IL) fitted with a Leica DFC 320 Digital Camera system. The diameters of the microcapsules were calculated as the average of 20 beads randomly selected with the aid of a dissecting microscope (Wild Heerbrugg, Switzerland) and the total number of cells released from 30 ± 2 microcapsules was evaluated using the trypan blue exclusion assay.

To examine the surface morphology of the microcapsules, scanning electron microscopy (SEM) was performed after fixing cell free beads with 2.5% glutaraldehyde in cacodylate buffer. The beads were processed through critical point drying using liquid carbon dioxide as transitional fluid and coating with carbon for viewing on Leo 1530 Field Emission Scanning Electron Microscope.

Table 1
The air-droplet generator parameters used in generating the homogenous microbeads

Parameter	Settings
Length of alginate needle shaft	18 mm
Inner diameter of needle	0.311 mm
Outer diameter of needle	0.518 mm
Pressure on alginate in the syringe	20 kPa
Air-flow rate	1.5 L/min

Isolation of adipose-tissue stromal cell cultures. ATSCs were isolated from 12 weeks old, green fluorescent protein (GFP) transgenic mice using a modification of published protocols [4,5]. Briefly, aseptically harvested adipose tissue was finely minced followed by incubation with collagenase type I at 37 °C for 45 min. Floating adipocytes were separated from the stromal fraction by centrifugation at 356*g* for 7 min. Cells from the stromal fraction were plated in a T75 flask at a density of 5000 cells/cm^2 and cultured with DMEM supplemented with 10% heat-inactivated FCS, 1× penicillin/streptomycin, and 2 mM L-glutamine in a controlled atmosphere of 5% CO_2, 95% humidified air, and at 37 °C. Non-adherent cells were removed after 24 h and adherent ATSCs referred to here as passage "0" were detached with 0.025% trypsin/0.01% EDTA after four days and re-plated. Passage 1 cells were used for this study.

ATSC encapsulation and culture. ATSCs were suspended in sterile-filtered alginate solution (1.5% w/v) at a concentration of 1×10^6 cells/mL. This alginate/cell mixture was converted into microcapsules with the droplet generator as described above. Microencapsulated and free ATSC, seeded approximately at equal cell density as determined during pilot study (4×10^3 cells as per replicate well respectively), were cultured for 21 days in complete culture medium supplemented with osteogenic stimulating factors consisting of 100 nM Dex, 50 mg/mL ascorbate-2-phosphate, and 10 mM β-GP.

Cell viability assay. Evaluation of cell viability was conducted as previously reported [17,18]. In the present study, tests were carried out weekly on both experimental groups for 21 days starting on day 1 and results were compared. Eight replicate wells of the 96-well plates (each containing 35 ± 2 microcapsules or equivalent number of 2D plastic surface cell cultures) of the three groups were evaluated at each time point. A predetermined linear calibration curve between optic density (OD) and cell concentrations was used to determine viable cell numbers.

Alkaline phosphatase (ALP) assay. Cells from microcapsules (35 ± 2 microcapsules) were prepared for ALP assay using protocols described by Weber et al. [19]. After two washings in PBS, microcapsules were incubated at 37 °C in a 50 mM EDTA-solution (pH 7.0) for 2 min to release cells. Cells from monolayer cultures were released by washings with cold PBS followed by two cycles of freezing and thawing and then gentle scraping with a cell scraper.

Aliquots of released cells were counted and sample was subsequently lysed with 20 μL of 0.1% Triton X-100. ALP activities of lysates were assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenylphosphate using the ALP kit according to the manufacturer's instructions. 200 μL samples from each group were analyzed with an ELISA-reader (SPECTRAmax GEMINI microplate) at 405 nm. ALP activity was normalized to total cellular protein determined by the Bradford protein assay protocol [17] and expressed as ng/mg protein/cell.

Histology. Calcified extracellular matrix deposition in the monolayer cell cultures was visualized by von Kossa staining. Cultures were rinsed twice in PBS, fixed with 4% paraformaldehyde for 20 min, and rinsed three times in double distilled water (ddH_2O). The cells were stained with freshly prepared 2% silver nitrate in a dark environment for 30 min followed by washes and exposure to light for 10 min. The reaction was terminated by rinsing thoroughly with ddH_2O.

For ALP staining, the azo-dye method was employed by using naphthol ASBI phosphate, and fast red salt as the substrate.

RT-PCR. Reverse transcription-linked polymerase chain reaction (RT-PCR) was used to analyze gene expression of cells after 14 days culture in osteogenic media. Primers for the osteoblast related gene, osteocalcin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), generally proportional to cell number, were analyzed. Primer sequences used and conditions for the PCR are shown in Table 2.

Statistical analysis. Each data point represents the mean of at least eight replicates. Student's *t* test (assuming equal variances) was performed to determine the statistical significance of data obtained from both experimental groups. A value of $P < 0.05$ was considered significant.

Table 2
Specific primers for PCR amplification listed with expected fragments size and optimal annealing temperature

Gene	Primer sequence	Product size (bp)	Annealing (°C)	Extension (°C)	Cycles
OCN	F: AGGGAGGATCAAGTCCCG R: GAACAGACTCCGGCGCTA	226	80	72	42
GAPDH	F: ACCACAGTCCATGCCATCAC R: TCCACCACCCTGTTGCTGTA	358	62	72	42

Expression of osteocalcin mRNA was demonstrated by RT-PCR.

Results

Cell viability

Morphology of monolayer cells remained fibroblastic while the encapsulated cells appeared as rounded cluster-like cells in the course of osteoinduction (Fig. 1A–D). Initial rapid proliferation of cells was observed in both groups with cell proliferation significantly higher in encapsulated group than monolayer group (Fig. 2). Cell number in encapsulated group increased from 3.9×10^3 cells on day 1 to 7.8×10^3 on day 7 in the microcapsules. Similarly, cell numbers in monolayer culture increased from 3.8×10^3 on day 1 to 6.8×10^3 on day 7. Subsequently, there was no significant difference in cell numbers between days 7 and 14 in both groups. However, cell numbers decreased significantly on day 21 in the monolayer group. In contrast, encapsulated ATSC showed no significant difference in cell numbers between day 7 and 21. Both experimental groups showed significantly higher number of cells on day 21 compared

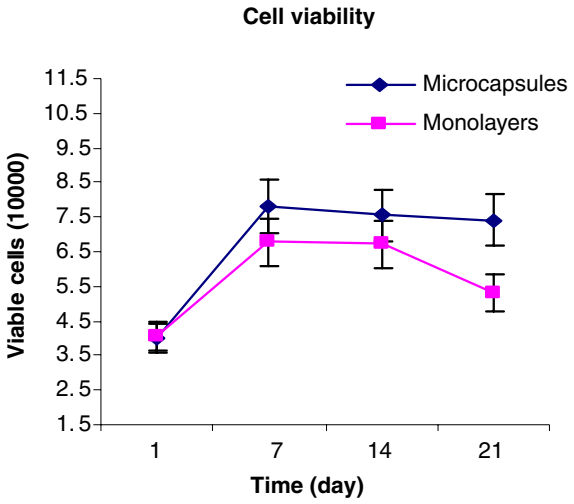

Fig. 2. Proliferation of ATSC monitored for 21 days. A predetermined linear calibration curve between optic density (OD) and cell concentrations was used to determine viable cell numbers.

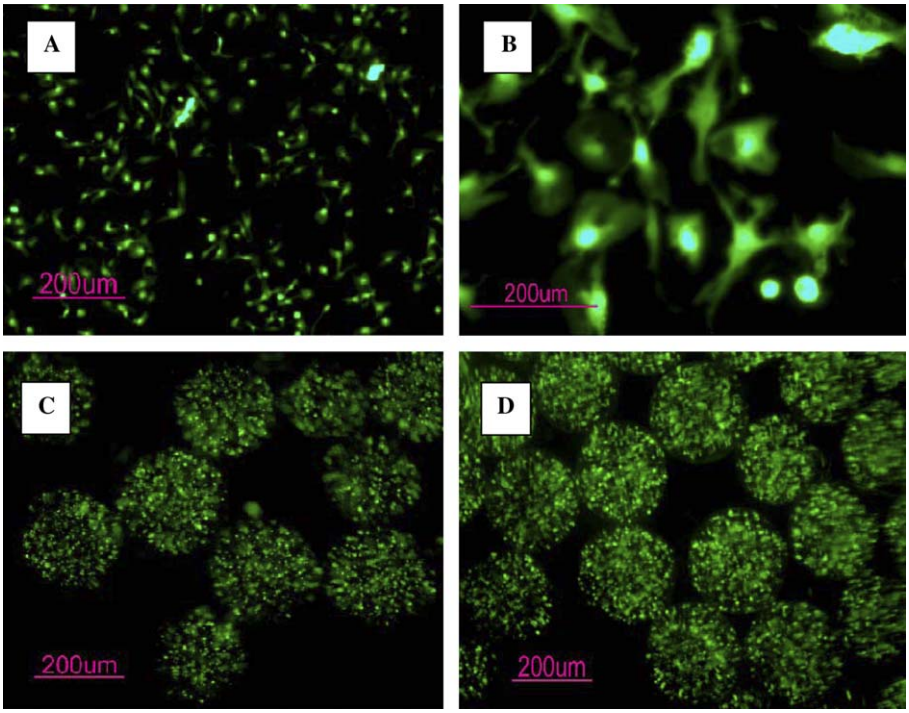

Fig. 1. Fluorescence microscopic images ATSC from GFP mice cultured either on monolayer (A, B) or entrapped in alginate microcapsules (C, D) after 24h (A, C) and 7 days (B, D) osteogenic induction media. Fibroblast like cells were observed in monolayer while microencapsulated cells remained rounded in shape during the course of osteoinduction.

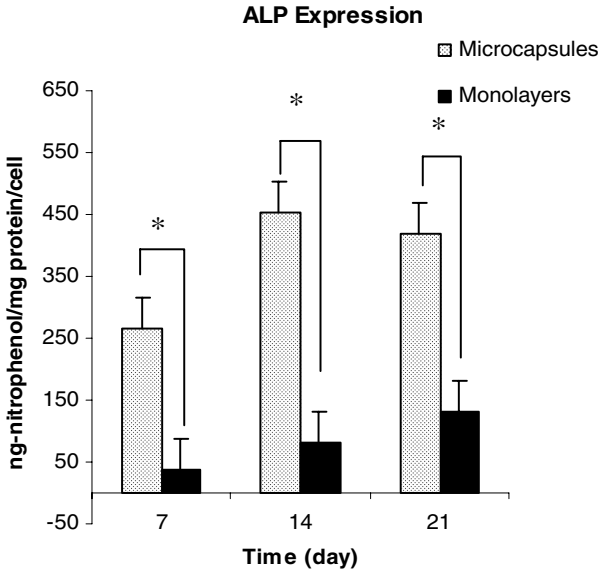

Fig. 3. Expression of bone specific ALP activity from ATSC in osteogenic induction medium in both monolayer and alginate encapsulated culture. ALP activity was normalized to total cellular protein determined and expressed as ng/mg protein/cell. $*p < 0.05$.

to day 1 representing excellent proliferation and viability in both groups.

ALP differentiation

Specific ALP levels were detectable on day 7 in both groups with encapsulated cells expressing significantly higher levels than 2D monolayer cell cultures (Fig. 3). Encapsulated ATSCs showed 6.9, 5.5, and 3.2 times higher expression level of ALP on days 7, 14, and 21, respectively, when compared with monolayer cultures ($P < 0.01$).

RT-PCR

The expression level of murine osteocalcin mRNA in osteogenic medium increased with time in both the encapsulated cells and monolayer cultures (Fig. 4). Microencapsulated cells showed an enhanced expression level of osteocalcin mRNA when compared with monolayer cultures over the course of 21 days. Osteocalcin was detected in the microcapsule group after 14 days of osteogenic induction and its expression had appreciably increased after 7 days of additional induction (Fig. 4). In contrast, osteocalcin mRNA of monolayer cultures were only detectable on day 21.

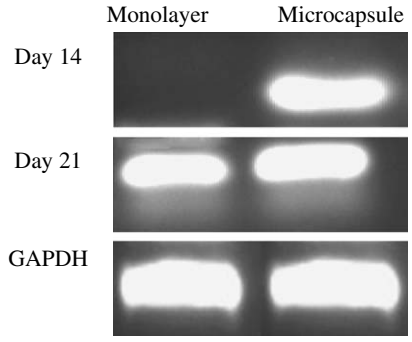

Fig. 4. RT-PCR analysis of the expression of an osteogenic specific gene, osteocalcin on day 14 and 21. Primers of GAPDH were used as a control.

Discussion

The use of calcium cross-linked sodium alginate microcapsules to deliver cells and other biological factors has been overwhelmingly explored in recent times principally because of their biodegradability and immunologic inertness [8–12]. However, the osteogenic behavior of adipose tissue stromal cells (ATSCs) confined in alginate microcapsules is yet to be reported as far as we know. Our results clearly demonstrate that the alginate microenvironment supports cell viability, is conducive for osteogenic differentiation, and maintains differentiated cellular function. Furthermore, the findings presented here indicate an enhancement of proliferation and osteogenic differentiation

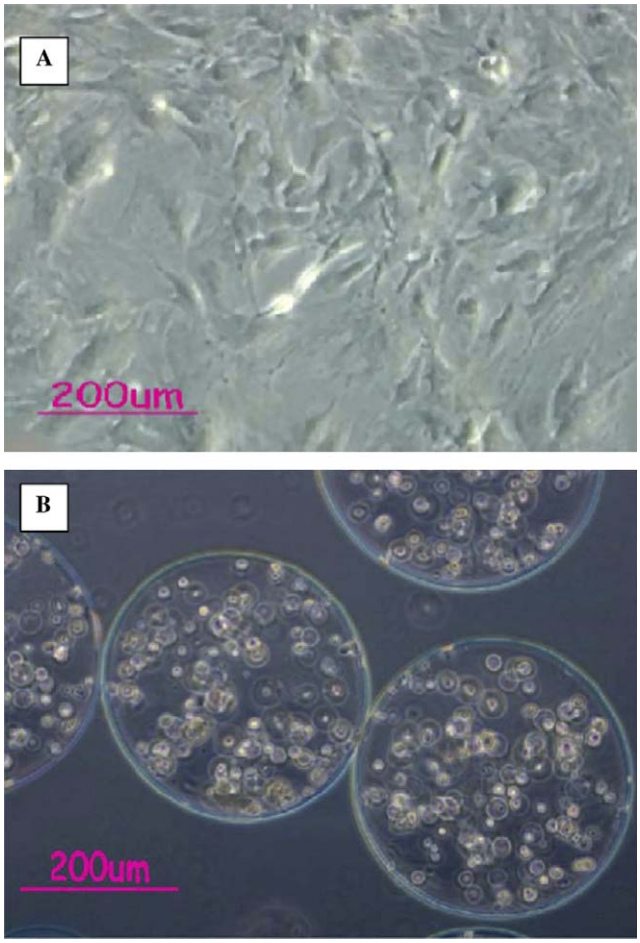

Fig. 5. Brightfield microscopy day 21: ATSC culture in osteoinductive medium on (A) monolayer and (B) microcapsules. Monolayer cells are broad. Microencapsulated cells are rounded and in clusters. Notice intact microcapsules.

of murine ATSC seeded at low density in calcium cross-linked solid core, sodium alginate microcapsule matrix made up of intermediate guluronic alginate and cultured in the presence of osteoinductive factors. We observed that while primary ATSC seeded at low density in alginate microcapsules proliferated in clusters or clumps and maintained a rounded or cuboidal morphology during the course of osteoinduction, cells on monolayer plastic cultures remained fibroblastic in morphology (Fig. 5A and B). Encapsulated cells were characterized by significant osteogenic activity demonstrated by a high expression of alkaline phosphatase and osteocalcin mRNA (Figs. 3 and 4). The expression of these early markers of osteoblast related factors suggests that the cells were undergoing osteogenic differentiation despite encapsulation. In addition, our findings indicate that ATSCs in microcapsules matrix attained a significantly higher population of viable cells during osteoinduction compared to monolayer cultured cells suggesting that the alginate microenvironment was a better environment for cellular and metabolic activity than the 2D monolayer plastic tissue culture surface during the 21-day osteoinduction. In contrast to encapsulated cells, ATSCs in monolayer cultures exhibited a fibroblast-like morphology even after 21 days of osteoinduction (Fig. 5A and B), with moderate morphological changes becoming broad and flat. This prompted further in vitro, phenotypic characterization of monolayer cultures with von Kossa and ALP staining. Monolayer ATSCs exhibited increasing level of osteogenic phenotype on osteoinduction as demonstrated by increasing deposition of mineralized matrix deposits on von Kossa and ALP staining (Fig. 6A–D).

The finding that encapsulated anchorage-dependent cells could proliferate inside alginate microcapsule was earlier reported by Bunger et al. while working on the mouse fibroblastic cell line, L929 [18]. It could be that the microcapsule alginate matrix provides a 3D microenvironment for cells to interact and grow faster than 2D monolayer. Three key factors contributing to the observed excellent cell viability and proliferation in alginate microcapsules are: (1) the small size of the microcapsule (250 μm ± 50 μm); (2) interconnecting fissures ensuring a porous matrix and enhancing circulation of nutrients and secreted biological products (Fig. 7A and B); and (3) the 3D microenvironment providing a niche for cell growth [10,20,21].

Previous studies have noted that suitable scaffolds for bone tissue engineering must have a porous and interconnected pore structure to ensure the circulation of nutrients and therefore provide a microenvironment that is biologically conducive for cell proliferation and growth [22,23]. SEM examination of our solid core, alginate microcapsules

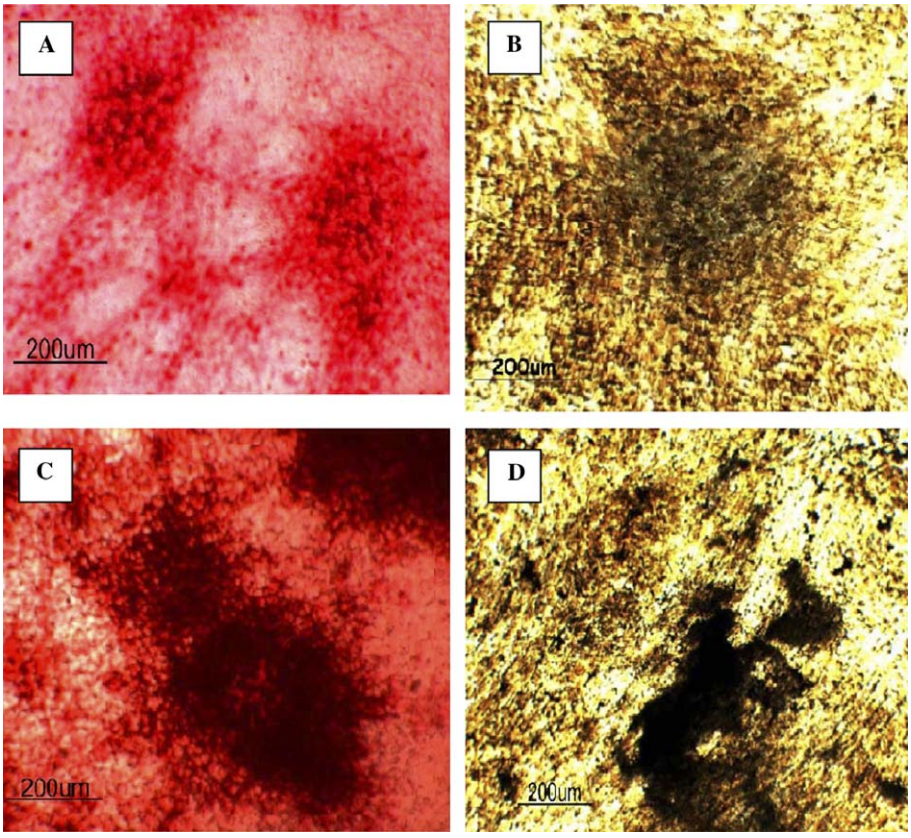

Fig. 6. Phenotypic characterization of ATSC on monolayer culture using ALP (A,C) and von Kossa (B,D) staining. Notice increasing deposition of mineralized matrix (dark stain) between day 7 (A,B) and day 21 (C,D).

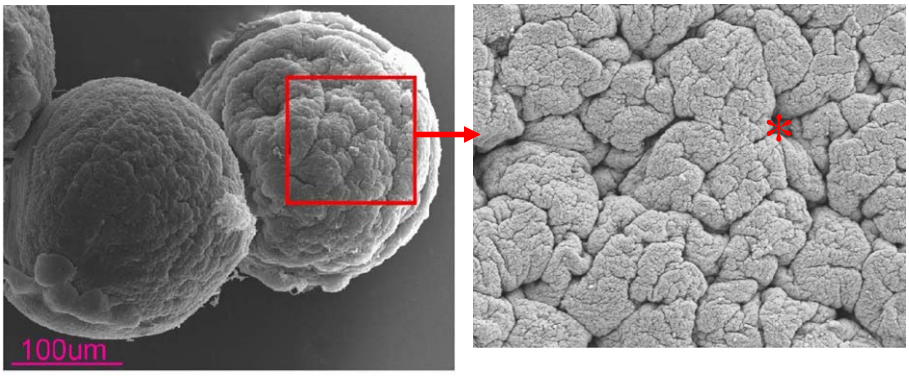

Fig. 7. Scanning electron microscopy of solid core calcium gelled sodium alginate microcapsules. Notice the multiple fissures on the surface of the microcapsules (star).

(as described in Materials and methods) indicates that the surface topography of these biodegradable hydrogels possess numerous interconnecting clefts and fissures (Fig. 5) enabling the circulation of nutrients and secreted products. Coating with polycations such as poly-L-lysine [16,17], and chitosan [23] has been widely reported to improve the immuno-isolating and mechanical properties of alginate microcapsules. The most widely studied alginate coating material is PLL. However, PLL coated microcapsules have been reported to elicit inflammatory response with intense aggregation of giant mononuclear cells at sites of implantation [24]. In addition, recent findings suggest that uncoated alginate microcapsules could also exhibit remarkable immuno-isolation of non-autologous cells [14,25]. Furthermore, multipotent mesenchymal cells are considered to be immune-privileged [26], therefore the need for immuno-isolation of these cells was not thought to be paramount in this study and coating was not performed.

Alginate hydrogel discs have been reported to inhibit the proliferation and growth of several different cells when these cells are either grown on the surface as 2D monolayer seeding or incorporated into the matrix of the gel [27]. This is in contrast to our findings. Possible explanation for these differences is the size and thickness of the scaffolds used as thicker matrix can impede perfusion of nutrients and secreted proteins [22]. In addition, the composition and grade of alginate employed play key role in the survival of cells in alginate. Wang et al. [28] previously showed that the ability of calcium cross-linked alginate to act as a substrate for rat marrow cell proliferation depended on its composition and purity. Furthermore, our pilot study suggests that these gel discs may have a lower mechanical stability in vitro than the microcapsules (unpublished) and these make 2D alginate culture a poor choice as control in this study.

In this experiment, ATSCs were used because they are reported to possess an excellent ability to proliferate and differentiate into different mesenchymal tissue lineage under appropriate conditions and relatively easy to derive on a larger scale [4].

Conclusion

This study shows that purified calcium cross-linked, solid core alginate microcapsules with intermediate guluronic acid content were able to act as substrate and support the proliferation and osteogenic differentiation of murine derived adipose-tissue stromal cells. We have demonstrated that these microcapsules are superior for murine ATSCs proliferation and osteogenic differentiation when compared to 2D monolayer plastic tissue culture surface. Based on the use of sodium alginates as cell encapsulation materials, this work demonstrates that it has potential to act as an injectable, cell immobilizing, 3D scaffold in bone tissue engineering. Further in vivo studies will be needed to determine if these scaffolds can be used to sustain survival of osteogenic cells over a desirable period of time in vivo.

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