Seeding cells into needled felt scaffolds for tissue engineering applications

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Abstract: Tissue engineering methods are under development that will enable the repair or replacement of a variety of tissues, including articular cartilage and bone. To engineer functional tissue it is necessary that scaffolds initially be seeded with a large number of cells distributed evenly throughout the scaffold structure. It previously has been shown that, compared to static seeding conditions, seeding scaffolds under dynamic conditions facilitates high seeding densities and even distributions of cells (Li et al., Biotechnology Progress 2001;17:935–944). The efficiency of seeding HOSTE85 cells and bovine chondrocytes into needled felt scaffolds following agitation at different speeds was determined. Seeding efficiency was determined using the Hoechst 33258 assay, and cell viability was assessed using the Alamar Blue™ assay. The distribution of cells within the scaffolds was imaged using scanning electron microscopy. It was found that the optimum seeding conditions varied for HOSTE85 cells and bovine chondrocytes, with different agitation speeds leading to different seeding efficiencies, cell viabilities, and distributions of cells within scaffolds. The optimum agitation speeds for seeding a high number of viable cells into scaffolds so that they were arranged evenly were 300 rpm for HOSTE85 cells and 200 rpm for bovine chondrocytes. © 2003 Wiley Periodicals, Inc. J Biomed Mater Res 66A: 425–431, 2003

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INTRODUCTION

Musculoskeletal injuries affect one in seven Americans, causing chronic pain, severely reducing the person’s quality of life, and costing the nation approximately $254 billion per year.¹ Damage to musculoskeletal tissues, such as articular cartilage and bone, may be the result of trauma, for example sports or road traffic accidents, or it may occur as a result of a disease, such as osteoarthritis.² It has been estimated that in excess of 40 million people in the United States suffer with arthritis each year.³ Tissue engineering methods are being developed that will allow the repair or replacement of such diseased or damaged tissues.⁴

Currently, many approaches for the engineering of organs and tissues are under development. One strategy is to take a biopsy of tissue, isolate the cells by enzymatic digestion, and then expand them in culture. The cells are then seeded into a suitable scaffold structure that will support the proliferating cells. The cell-seeded construct must then be cultured under appropriate conditions to allow extracellular matrix formation and tissue regeneration.⁵

There are many important parameters that must be optimized to enable the formation of functional tissues. It is important that the scaffold employed supports cell attachment, extracellular matrix and tissue formation, and that the constructs initially be seeded with a high number of cells that are distributed evenly throughout the entire scaffold.⁶ For example, when tissue engineering articular cartilage, a high seeding density is necessary to prevent fibrous tissue formation.⁷ It also is necessary for cells to be distributed evenly throughout the scaffold structure for normal tissue formation.⁸

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It previously has been reported that higher seeding densities and more uniform distributions of cells in scaffolds can be achieved using dynamic rather than static seeding methods. This technical note describes for two different cell types the relationship of cell seeding and cell viability with the rate at which cell-seeded scaffolds are agitated during the initial seeding process.

**MATERIALS AND METHODS**

**Materials**

Poly(ethylene terephthalate) (PET)-needled felt scaffolds (45 mg/cm²) were manufactured and supplied by Smith and Nephew (York, UK). Lower legs from 30-month-old cows were obtained from a local abattoir. HOSTE85 cells were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK). Dulbecco's modified Eagle's medium (DMEM) and gentamicin were from GIBCOBRL Life Technologies (Glasgow, Scotland). Pronase was from BDH (Lutterworth, UK). Alamar Blue™ was from Serotec (Kidlington, UK). Glutaraldehyde and osmium tetroxide were from Taab Laboratories (Berkshire, UK). All other reagents were purchased from Sigma (Poole, UK).

**Culture of HOSTE85 cells**

HOSTE85 cells were cultured in DMEM supplemented with 10% (v/v) fetal calf serum (FCS), 1% (v/v) nonessential amino acids (NEAA; ×100), 2 mM of L-glutamine, 100 units/mL of penicillin, 0.25 μg/mL of amphotericin B, 0.1 mg/mL of streptomycin, and 0.15 mg/mL of ascorbic acid (HOSTE85 media). When confluent, a cell suspension was obtained by enzymatic digestion with 0.25% trypsin/0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate-buffered saline (PBS; pH 7) and diluted to 4×10⁶ cells per mL in HOSTE85 media.

**Isolation of chondrocytes**

Full-thickness cartilage was aseptically removed from the metacarpophalangeal joint of freshly slaughtered 30-month-old cows. Chondrocytes were isolated, as previously described by Archer et al. Briefly, cartilage pieces were washed in PBS and finely diced. Chondrocytes were obtained by enzymatic digestion with pronase (700 units/mL of activity) for 1 h and collagenase (300 units/mL of activity) for 3 h at 37°C and 5% CO₂. Chondrocytes were diluted to 4×10⁶ cells per mL in DMEM supplemented with 10% (v/v) FCS, 1% (v/v) NEAA (×100), 2 mM of L-glutamine, and 50 μg/mL of gentamicin (chondrocyte media).

**Needled felt scaffolds**

Sheets of the PET needled felt material were cut into discs 8 mm in diameter and 3 mm in thickness. Scaffolds were autoclaved at 120°C for 20 min and presoaked in either chondrocyte or HOSTE85 media overnight at 37°C and 5% CO₂ prior to seeding.

**Seeding cells into scaffolds**

Scaffolds were placed in triplicate for biochemical analysis and in duplicate for image analysis in 24-well plates. One mL of media containing 4×10⁶ cells was added to each well and pipetted through the scaffold matrix. The plates were immediately placed on one/quarter of the orbital shaker (IKA³, Schüttler MT54, Germany) with a balance-plate placed diagonally opposite and agitated at 0, 100, 200, 300, or 400 rpm (for chondrocytes) or 100, 200, 300, or 400 rpm (for HOSTE85 cells) overnight in an incubator at 5% CO₂ and 37°C. Control scaffolds were agitated in the same way but with media alone. The plates were placed in the same position on the shaker for every agitation speed.

**Determination of cell viability**

The viability of cells within the scaffolds was determined with the Alamar Blue™ assay, using a method adapted from that reported by Fields and colleagues. The scaffolds were removed from culture, washed three times in PBS, and incubated with 10% Alamar Blue™ in Hank’s balanced salt solution (HBSS) without phenol red (pH 7) for 90 min. Aliquots (200 μL) of Alamar Blue™/HBSS were placed in a 96-well plate in triplicate, and the fluorescence was measured at an excitation wavelength of 530 nm and an emission wavelength of 595 nm using a fluorescence plate reader (F2 Microplate Fluorescence, Absorbance and Luminescence System, Labtech, UK).

**Determination of DNA content**

For the determination of the number of cells within scaffolds, the scaffolds were washed three times in PBS, frozen, and lyophilized. Pellets of 8×10⁶ chondrocytes and HOSTE85 cells also were frozen and lyophilized. The scaffolds and cell pellets were digested overnight in 1 mL of papain (1.06 mg/mL) at 60°C, as described by Kim and colleagues. Serial dilutions of the digested cell pellets were prepared as standards (0, 1.25×10⁵, 2.5×10⁵, 5×10⁵, 1×10⁶, 2×10⁶, 4×10⁶, and 8×10⁶ cells/mL). The amount of DNA within the controls, sample triplicates, and standards was determined using Hoechst 33258 dye. Fluorescence was measured at an excitation wavelength of 355 nm and an emission wavelength of 460 nm on a fluorescence plate.
The amount of DNA was related to cell numbers using a standard curve of cell number versus average fluorescence (nm).

**Scanning electron microscopy**

Scaffolds were prepared for scanning electron microscopy, as described in a method by Robinson and Gray. Briefly, scaffolds were removed from culture, washed three times in PBS, and fixed overnight in 3% glutaraldehyde in PBS at 4°C. The scaffolds were washed three times in PBS and fixed further in 1% osmium tetroxide for 2 h. The scaffolds then were washed three times in distilled water, dehydrated with increasing concentrations of ethanol (25, 50, 70, 90, 95, 100%, v/v), and dried with hexamethyldisilazane (HMDS). The scaffolds were placed on carbon-coated stubs and sputter-coated with gold. They then were imaged using a scanning electron microscope (Philips IL505).

**Statistics**

The statistical significance of the results was assessed using GraphPad InStat, v. 3.00 (GraphPad Software Inc, San Diego). One-way analysis of variance (ANOVA) and the Tukey-Kramer multiple comparisons post-test were performed.

**RESULTS AND DISCUSSION**

**HOS cells**

Seeding efficiency and cell viability

The effect of agitation speed on the number of HOSTE85 cells seeded into needled felt scaffolds is shown in Figure 1(a). Increasing the rate at which scaffolds were agitated led to an increase in the number of cells within the scaffolds. The relative total viability of HOSTE85 cells in needled felt scaffolds seeded at each of the speeds is shown in Figure 1(b). No significant difference in relative viability was detected for any of the agitation speeds. The relative viability per cell can be determined by normalizing the viability reading with respect to the number of cells present in the scaffolds and is shown in Figure 1(c). A decrease in relative viability per cell with increasing agitation speed was observed. For all control scaffolds, no DNA or cell viability was detected.

Spatial arrangement

Representative scanning electron micrographs in Figure 2 show the arrangement of HOSTE85 cells within needled felt scaffolds agitated at 100 and 300 rpm. Following agitation at 100 rpm, few HOSTE85 cells were visible within the scaffolds [Fig. 2(a,b)]. Agitation at 300 rpm caused a greater number of HOSTE85 cells to be retained within the scaffolds, and cells were distributed evenly throughout the entire scaffold structure, spreading between fibers, as shown in Figure 2(c,d). Agitation at 400 rpm led to a further
increase in the number of HOSTE85 cells present, but
the cells were concentrated in the center of the scaffold
and normal cell morphology was compromised (im-
gages not shown).

Increasing the rate of agitation during seeding in-
creases the number of HOSTE85 cells retained within the
scaffolds, but it also results in a decrease in the viability
of the cells. Agitating the scaffolds at 400 rpm also led to
an uneven distribution of cells within the scaffolds and
normal cell morphology was compromised.

Chondrocytes

Seeding efficiency and cell viability

The seeding efficiency with which chondrocytes
were seeded into needled felt scaffolds following
shaking at 0, 100, 200, 300, and 400 rpm is shown in
Figure 3(a). Increasing the speed of agitation from 0 to
200 rpm led to an increased number of cells within the
scaffold, but, surprisingly, increasing the speed fur-
ther resulted in a reduction in the number of cells
retained in the scaffolds. The relative overall viability
of chondrocytes seeded into needled felt scaffolds is
shown in Figure 3(b).

The scaffolds seeded statically showed statistically
lower relative viability than the scaffolds agitated at
the other speeds. The relative viability per cell for
chondrocytes in each scaffold was calculated by divid-
ing the average viability readings by the number of
chondrocytes in each needled felt scaffold [see Fig.
3(c)]. No significant difference among the relative vi-
abilities of individual chondrocytes in needled felt
scaffolds agitated at different speeds was detected. For
all control scaffolds, no DNA or cell viability was
detected.
Figure 4 shows representative scanning electron micrographs of chondrocytes in needled felt scaffolds agitated at 0, 200, and 400 rpm. Culturing the scaffolds overnight at 0 rpm caused only a few chondrocytes to be retained within the needled felt scaffolds [Fig. 4(a,b)]. Agitating the scaffolds at 100 rpm resulted in an increase in the number of chondrocytes both at the surface and in the center of the scaffolds (images not shown). Further increasing the speed of agitation to 200 rpm caused a great increase in the number of chondrocytes, and they were distributed evenly throughout the scaffolds [Fig. 4(c,d)]. Agitating the scaffolds at 400 rpm [Fig. 4(e,f)] lead to a less even distribution of cells throughout the scaffolds, with the chondrocytes concentrated in the centers of the scaffolds.

Agitating needled felt scaffolds containing bovine chondrocytes at 200 rpm produced optimum seeding. At this agitation speed, a high density of chondrocytes was seeded into scaffolds and evenly distributed throughout them without their viability being compromised.

CONCLUSIONS

Tissue engineering methods are under development that will allow the formation of functional tissues and organs. The density with which cells initially are seeded into scaffolds and their arrangement within the scaffolds are important parameters in tissue engineering. These factors affect cell proliferation, differentiation, and migration, and ultimately they affect the quality of the engineered tissue.6

The agitation speed required for obtaining optimum seeding for HOSTE85 cells in needled felt scaffolds, as determined from this study, is 300 rpm. At this speed a large number of cells are retained within the scaffolds, they are distributed evenly throughout, and their morphology is not compromised. The optimum seeding efficiency for bovine chondrocytes in needled felt scaffolds in this study was achieved by agitating the scaffolds at 200 rpm. Chondrocytes were distributed evenly throughout the scaffolds and the viability of each chondrocyte was not compromised.

The findings of this study support previous studies that have shown improved seeding of cells into scaffolds using dynamic rather than static seeding methods.6,8 The optimum seeding conditions for the two cell types were different, as shown by the efficiency with which cells were seeded into the scaffolds and the arrangement of cells within the scaffolds. It is unknown why the cells respond differently to agitation; however, it is postulated that it may be due to differences in cell size or density. A study of this nature therefore is vital prior to commencing tissue engineering work in order to determine the optimum parameters for seeding scaffolds.
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References


Figure 4. Arrangement of chondrocytes within needled felt scaffolds shaken at 0, 200, and 400 rpm. The distribution of cells at the surface of scaffolds agitated at (a) 0 rpm, (c) 200 rpm, and (e) 400 rpm can be seen. The distribution of cells within the scaffolds agitated at (b) 0 rpm, (d) 200 rpm, and (f) 400 rpm also are shown. The images are representative examples (for each speed n = 2).