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Optimization of chondrocyte isolation and characterization for large-scale cartilage tissue engineering

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ABSTRACT

Background: Advancements in cartilage tissue engineering have the potential to ameliorate facial and joint reconstructive surgery as we know it. The translation of *in vitro* models of cartilage regeneration into clinical scenarios is the next phase of cartilage tissue engineering research. To engineer larger, more robust, and clinical relevant constructs, a great number of viable chondrocytic cells are needed. However, there is a paucity of literature concerning the most favorable method of chondrocyte isolation. Isolation methods are inconsistent, resulting in small yields and poor cell quality, and thus unreliable neocartilage formation. This study aimed to optimize the chondrocyte isolation protocol to give a maximum yield with optimal cell viability for the engineering of large cartilaginous constructs such as the human nose and ear.

Methods: We employed several enzymes (pronase, dispase, hyaluronidase, and collagenase), enzyme concentrations, and digest lengths to digest freshly harvested ovine nasoseptal cartilage. We used automated trypan blue live/dead staining, immunofluorescent labeling of CD44, collagenase II, collagenase I, and Aggrecan, and alamarBlue to assess cell yield and viability.

Results: Incubation length in enzymatic solutions had the greatest effect on cell viability, whereas concentrations of enzymes had a lesser effect. Isolated cells maintained their expression of chondrocyte-specific cell surface markers.

Conclusions: The optimum incubation period was 10 h using collagenase at a 0.2% (w/v) solution. An average of $1–1.5 \times 10^6$ cells could be harvested per gram of cartilage using this method.

1. Introduction

The absence of key craniofacial structures, namely the nose and ear, as a result of oncologic resection, trauma, or congenital malformation can cause a patient to experience social insecurity and severe depression [1]. Even with the advent of wax and silicone facial prostheses, patients report a feeling of incompleteness. For many years, surgeons have sought to reconstruct the nose and ear using autologous transplantation of cartilage from the contralateral ear or ribs,

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and bone from the hip [2]. Disadvantages of this method include postoperative scarring, pain, the risk of infection, and poor healing at the donor site. In addition, the cartilage harvested is often too little or too rigid to effect a perfect aesthetic and functional reconstruction [3].

The success of cartilage tissue engineering for the treatment of osteoarthritic and rheumatologic disease has prompted investigation into its application in engineering facial cartilage. Tissue engineering employs biological or synthetic scaffolds and complex cell differentiation techniques to regenerate damaged or lost tissue within the body. A sizeable amount of research has been conducted in developing whole or "off-the-shelf" tissue-engineered nasal and auricular cartilage constructs. However, to date, most attempts have been small-scale, producing mechanically immature tissue prone to resorption *in vivo* [2,4].

To engineer bigger and better constructs, large populations of viable chondrocytic cells are needed. The current zeitgeist is that autologous mesenchymal stem cells would be an ideal renewable cell source [5]. The application of this technology is limited by difficulties in stopping the differentiation of mesenchymal stem cells before they turn into osteoblasts (thereby making bone) [6–8]. There is also a tendency, if successfully differentiated, for these cells to produce immature and fragile neocartilage unsuitable for making three-dimensional constructs [9]. In light of these issues, many research groups continue to use mature adult chondrocytes isolated from harvested cartilage to advance the development of whole tissue–engineered constructs [10–12].

Cartilage is not the most cellular of tissues; only 5%–10% of its volume consists of cells. These cells, known as chondrocytes, sit in fluid-filled spaces known as lacunae, surrounded by a dense extracellular matrix (ECM) of collagenases, proteoglycans, and glycosaminoglycans (Fig. 1). During the breakdown of the dense ECM, chondrocytes are often exposed to harsh enzymes for prolonged periods of time. This reduces not only the final cell number, but also the viability and proliferative capacity of the cells. To compensate for this, many research groups have tried to increase cell number by passaging; however, the propensity of chondrocytes to dedifferentiate into fibroblasts over repeated passages is a major problem [13–15]. Ideally, for engineering large constructs that are to be in culture for a long time and to maintain consistency in tissue quality, huge numbers of cells with high viability and minimal passage number should be used [15].

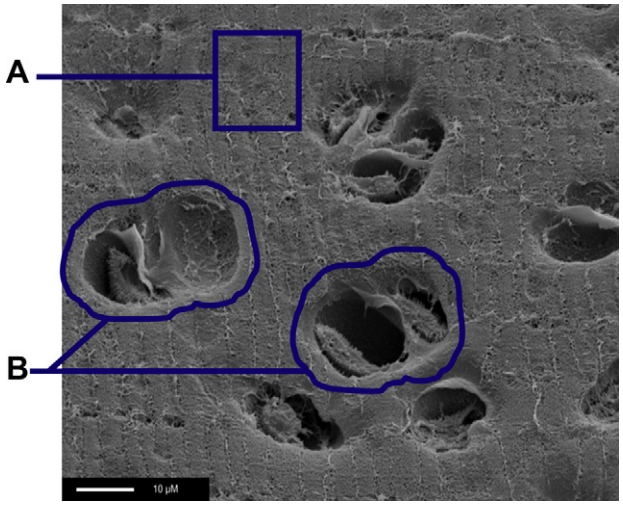

Fig. 1 – Scanning electron micrograph of a cross-section of ovine nasoseptal cartilage. (A) Cartilage ECM secreted by chondrocytes. (B) Chondrocytes in fluid-filled spaces known as lacunae. (Color version of figure is available online.)

Recent studies have shown vast discrepancies in chondrocyte isolation practice (Table 1). Concentrations and types of collagenase vary widely, as do the inclusion or exclusion of predigest stages and variations in the optimal length of digest. Such incongruous methodologies are doubtless responsible for inconsistent cell numbers, function, and viability. As such, this study aimed to optimize chondrocyte isolation practice according to stages of digest, length of digest, and concentration of enzymes. We assessed the cell number and viability of the cells after digest and characterized the cell yield, because the number of true chondrocytes in each harvest has not yet been investigated in detail. We also optimized the seeding density of these cells, because few studies have investigated this, although it is crucial for understanding key cell behaviors over prolonged culture periods.

2. Material and methods

2.1. *Optimization of chondrocyte isolation*

2.1.1. *Cartilage harvest*

We harvested normal noses from 40 male sheep with a mean age of 15 mo shortly after slaughter, and transported them in unsupplemented Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, UK) at 4°C to our laboratory. We harvested nasoseptal cartilage and carefully dissected away the perichondrium. We washed the cartilage three times with sterile phosphate-buffered saline (PBS) (Gibco, UK) and minced it into 1-mm^3 pieces. We washed the minced cartilage again and weighed it out into 1 g/sample.

2.1.2. *Evaluation of predigest*

The samples underwent a 1-h predigestion stage with an enzyme, either 0.8 U/mg Dispase (Sigma Aldrich, UK), 0.1% hyaluronidase (Sigma Aldrich), or 0.4% pronase (Roche, UK), dissolved in *N*-2-hydroxyhydroxyethylpiperazine-*N*′(2-ethanesulphonic acid) (HEPES)-buffered DMEM (Gibco, UK) supplemented with 50 U/50 μg/mL penicillin-streptomycin (Sigma Aldrich), 2.5 μg/mL amphotericin B, and 10% fetal bovine serum (FBS) (Gibco, UK) (Table 1). We filter-sterilized all enzymes by passage through a 0.22-μm filter. We also used a negative control of supplemented HEPES-buffered DMEM. We used 10 mL solution per gram of cartilage. We then incubated the cartilage pieces for 1 h at 37°C on a shaker at about 30–40 rpm. The cartilage fragments were then washed twice with PBS and subjected to collagenase II (Sigma Aldrich) 0.15% digests for 10 h. We dissolved the collagenase II in supplemented HEPES-buffered DMEM media and sterile-filtered it. We used 10 mL of this medium per gram of cartilage and incubated the cartilage at 37°C on a shaker without CO_2.

Table 1 – Variation in chondrocyte isolation protocol: Overview of some of the methods used to isolate chondrocytes for cartilage tissue engineering.

Species	Enzymes	Length of digest	Cell yield/g	Reference
Human	Collagenase II: 0.2%	16 h	4.45 ± 2.28 × 106	[18]
Human	Collagenase II: 0.08%	Overnight		[19]
Human	Collagenase II: 0.15%	22 h		[20]
Human	Collagenase II Trypsin/EDTA Hyaluronidase Tosyl-lysyl chloromethane	Overnight	<22% of available cells	[21]
Human	Deoxyribonuclease 1: 0.015% Hyaluronidase: 0.1% Collagenase II: 0.2%	18–36 h		[22]
Rabbit	Predigest with: Hyaluronidase: 0.05% Collagenase II: 0.2% Trypsin: 0.2%	1–2 h		[23]
Human	Trypsin Protease Hyaluronidase (varied concentrations)	Low-temperature method, 12 h	1.68×10^5/g	[17]
Sheep	Collagenase II: 0.3%	5–8 h		[24]

2.1.3. *Optimization of collagenase digest*

Once we established the optimum predigest condition, we optimized collagenase II digestion using different concentrations of collagenase 11 (0.1%, 0.15%, and 0.2%) for different lengths of time (6, 10, and 16 h) (Table 2). We made the digest media as stated above.

2.1.4. *Chondrocyte harvest and culture*

For all digests, we passed the triturate suspension through a 100-μm nylon cell strainer (BD Falcon, UK) to remove matrix debris, and added it to 40 mL DMEM/F-12 (1.1) with GlutaMAX (DMEM/F-12) (Gibco, UK) media, supplemented with 20% FBS, 1% penicillin-streptomycin and 1% amphotericin B. The 20% FBS helped neutralized enzyme activity and provide nutrients to the shocked cells. We centrifuged the filtrate at 2000 rpm for 10 min to pellet the cells. We discarded the supernatant, washed pelleted cells in sterile PBS, and resuspended them in supplemented DMEM/F-12 (20% FBS). We performed cell count and viability assessment using the Countess Cell Counter (Invitrogen).

We plated harvested cells into flasks in 20% FBS DMEM/F-12 supplemented media undisturbed for 3 d, in a humidified incubator at 37°C and 5% CO_2. This was to allow the cells a period of recovery before experimentation or cryopreservation. We seeded cells at a density of 1×10^6 cells per T-75 flask.

2.2. *Characterization of cells*

2.2.1. *Histology*

We seeded cells at a density of 1×10^4/T-25 flask. Before staining, we rinsed the cells in sterile PBS and fixed them in 2.5% (w/v) glutaraldehyde/PBS for 30 min, and rinsed them again with PBS. We used standard histological methods to perform Alcian Blue (Sigma Aldrich, UK) and Neutral fast red (Sigma, UK) staining to localize the glycosaminoglycan

Table 2 – Digest schedule: Comparisons made between different enzymatic digestion protocols, showing enzymes used, digestions times, and concentrations.

	Predigest*				Collagenase digest		
	Enzyme	Concentration	Time (min)		Enzyme	Concentration	Time (min)
1	Dispase	0.8 U/mg	60	5 (a) (b) (c)	Collagenase II	0.10% (w/v)	360 600 960
2	Hyaluronidase	0.1% (w/v)	60	6 (a) (b) (c)	Collagenase II	0.15% (w/v)	360 600 960
3	Pronase	0.4% (w/v)	60	7 (a) (b) (c)	Collagenase II	0.20% (w/v)	360 600 360
4	Control	–	60				

* All predigest experiment underwent additional collagenase II (0.15%) for 10 h.

content. We used Picro-Sirius red (Sigma, UK) stain to visualize collagenase distribution and orientation, and Safranin O (Sigma Aldrich, UK) to highlight the proteoglycan content. We performed visualization and photography using a Nikon (UK) TMS light microscope and Optem (UK) InfinityY2-1L imaging system.

2.2.2. *Immunofluorescence*

We characterized cells according to the expression of specific surface proteins. For CD44 (Lifespan Biosciences, UK), collagenase II, collagenase I, and Aggrecan (Millipore, UK), we characterized by immunofluorescence imaging using mouse host antibodies. We also used a secondary antibody of fluorescein isothiocyanate–conjugated anti mouse immunoglobulin G antibody along with a negative control of anti-mouse immunoglobulin G2b–fluorescein isothiocyanate conjugate. CD44 was conjugated to red fluorescent quantum dot (synthesized in our lab) and used for labeling. We used a Zeiss (UK) confocal microscope and C1 imaging software (UK).

2.3. *Optimization of seeding density*

2.3.1. *alamarBlue (AB)*

alamarBlue (Serotec, Kidlington, UK) is used for the quantitative measure of cell proliferation, cytotoxicity, and viability. Resazurin and resarfurin are used as colorimetric indicators of oxidation. These indicators respond by changing color according to changes in cell metabolism. The color change is measured at absorbances of 570 and 630 nm. The advantages of this assay are that it is soluble in media, stable in solution, and minimally toxic to cells, and produces changes that are easily assessed [16]. To optimize the seeding density, we cultured P0 cells for up to 14 d at the following densities: 1×10^3, 5×10^3, 1×10^4, 5×10^4, 1×10^5, 5×10^5 and 1×10^6. We added AB to cell culture media (CCM) at a concentration of 10% (v/v). At each assay time point, we removed the media from the wells, washed cells with 1 mL PBS, and added 1 mL AB/CCM to the wells. We used AB/CCM in wells with no cells as a negative control. After 4 h, we removed a 200-μL sample of the AB/media and measured the absorbance at 570 and 630 nm in a 96-well plate (Nunc, Thermo Fisher Scientific, UK) using the Fluoroskan AscentFL spectrofluorometer (Thermo Lifesciences, Basingstoke, UK).

2.3.2. *Lactate dehydrogenase assay (LDH)*

We measured LDH using a CytoTox 96s nonradioactive cytotoxicity assay kit (Promega, Southampton, UK). The amount of LDH released is measured using a 30-min coupled enzymatic reaction based on the conversion of a tetrazolium salt INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl tetrazolium chloride) into a red formazan product. The amount of coloration produced is directly proportional the number of lysed cells. We transferred 50-μL samples of CCM from each well to a 96-well plate (Thermo Fisher Scientific, UK). We added 50 μL of substrate mix to each well and covered the plate in foil. After a 30-min incubation period, we stopped the reaction by adding 50 μL stop solution (1 mol/L acetic acid). We then read the absorbance at 492 nm using an Anthos Microplate absorbance reader 2020, version 2 (Biochrom Ltd., Cambridge, UK).

2.4. *Statistics*

Data are presented as means ± standard deviation. We performed comparisons between groups using one-way and two-way analyses of variance along with Tukey's and Dunnett's multiple comparison post-tests. We performed statistical analysis using GraphPad Prism version 5.0 software (UK). $P < 0.05$ was considered statistically significant.

3. Results

3.1. *Evaluation of predigest*

After a 1-h digest period, there was no significant difference in the number of cells produced when we used hyaluronidase or pronase enzyme compared with the negative control (no enzyme). Treatment with dispase enzyme produced a significantly higher cell yield than in the control ($P < 0.0001$) ($n = 9$) (Fig. 2). Subjected to a continued digest for 10 h in 0.15% collagenase II, there was no significant difference in the cell yield between the control, dispase, and hyaluronidase enzyme. The number of cells produced in the pronase group was significantly less than the negative control ($P < 0.0001$) ($n = 9$) (Fig. 2). There was significant reduction in the viability of the cells from all experimental groups compared with the control ($P < 0.0001$) after the completed digest. Cells subjected

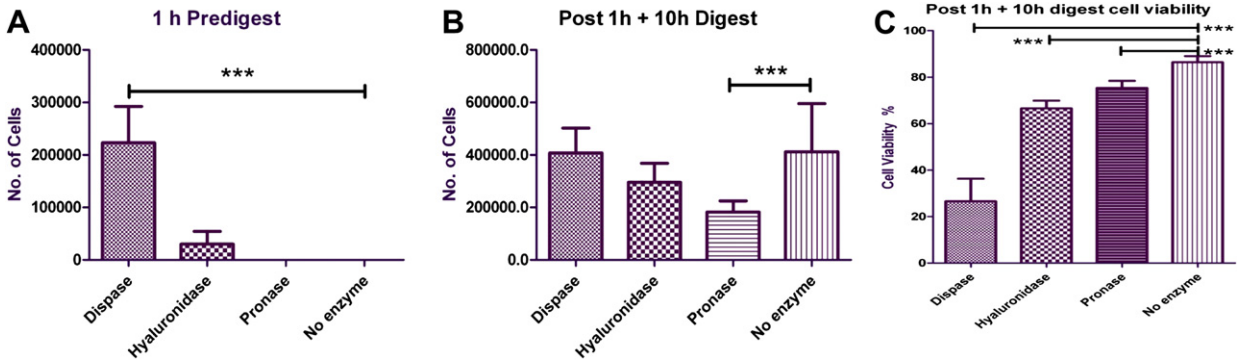

Fig. 2 – Results of predigest evaluation. (A) Cell yield after 1 h. (B) Cell yield after 1 h plus 10 collagenase II digest. (C) Cell viability (%). ***$P < 0.0001$. (Color version of figure is available online.)

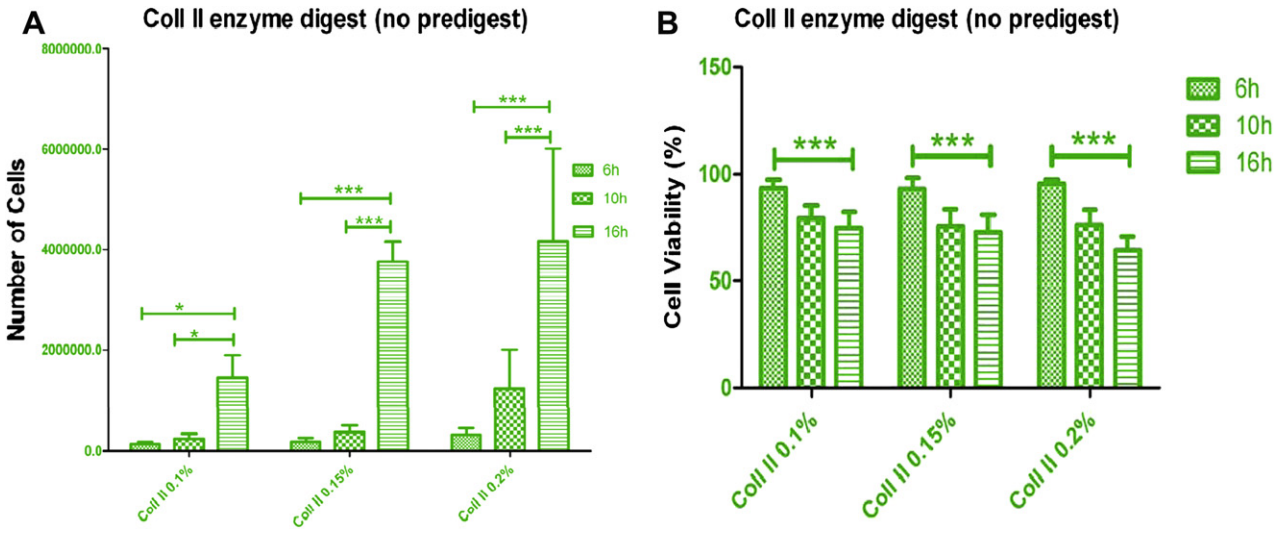

Fig. 3 – Collagenase digest optimization. (A) Cell yield. (B) Cell viability. *$P < 0.05$. ***$P < 0.0001$. (Color version of figure is available online.)

to dispase digest had the least viability of all experimental groups ($P < 0.0001$) ($n = 9$) (Fig. 2).

3.2. *Optimization of collagenase digest*

There was a significant increase in cell yield between 0.1% and 0.15% collagenase II. The increase seen between 0.15% and 0.2% at all three times was not significant. There was a significant increase in cell yield over time ($P < 0.0001$). We also deemed the interaction between variables of concentration and time to be statistically significant ($P < 0.001$) ($n = 5$) (Fig. 3). We did not consider the effect of concentration on cell viability to be significant, whereas time was a significant factor. The decrease in viability could be attributed to time at all three collagenase II concentrations ($P < 0.0001$) ($n = 5$) (Fig. 3).

3.3. *Cell characterization*

We performed immunofluorescence on harvested chondrocytes to investigate their expression of chondrocyte markers, CD44, collagenase II, collagenase I, and Aggrecan. Chondrocytes strongly expressed CD44 and collagenase II, as expected (Fig. 4A and B). There was also expression of Aggrecan and collagenase I, but this was comparatively reduced (Fig. 4C and D).

Over the 4-, 7-, and 14-d culture period, the cells' appearance took on a more rounded morphology (Fig. 5). The cells were confluent in T-25 flasks after a 14-d culture period, and there was an increase in the amount of staining of all three ECM proteins over that period.

3.4. *Optimization of seeding density*

The optimum seeding density for maintaining good cell viability for chondrocytes in a 24-well plate over a 10-d incubation period was 1×10^5 ($P < 0.001$) (Fig. 6). The cells in this well continuously increased over the 10-d incubation period, evidencing potential for continued growth over an extended culture period. Seeding at 5×10^5 had the highest peak viability at Day 7, but suffered a sharp decline in viability up until Day 10 ($P < 0.001$). There was no significant difference in the results of the lactate dehydrogenase assay.

4. Discussion

The isolation of chondrocytes from cartilage for tissue engineering research and therapeutics is a contentious issue. Each laboratory has a unique method for isolating chondrocytes, involving a number of different enzymes, incubation lengths,

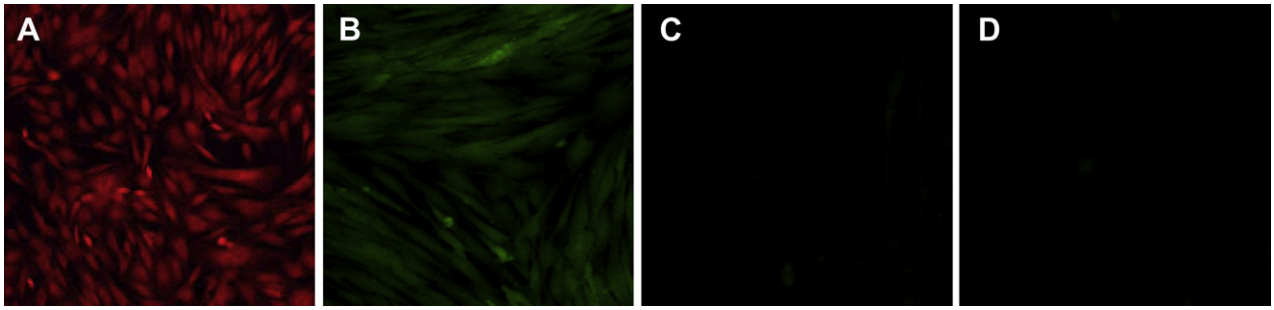

Fig. 4 – Immunohistochemistry of P2 chondrocytes seeded on tissue culture plastic in 24-well plates. (A) Quantum dot-labeled Cd44. (B) FITC-labeled collagenase II. (C) FITC-labeled collagenase I. (D) FITC-labeled Aggrecan. (Color version of figure is available online.)

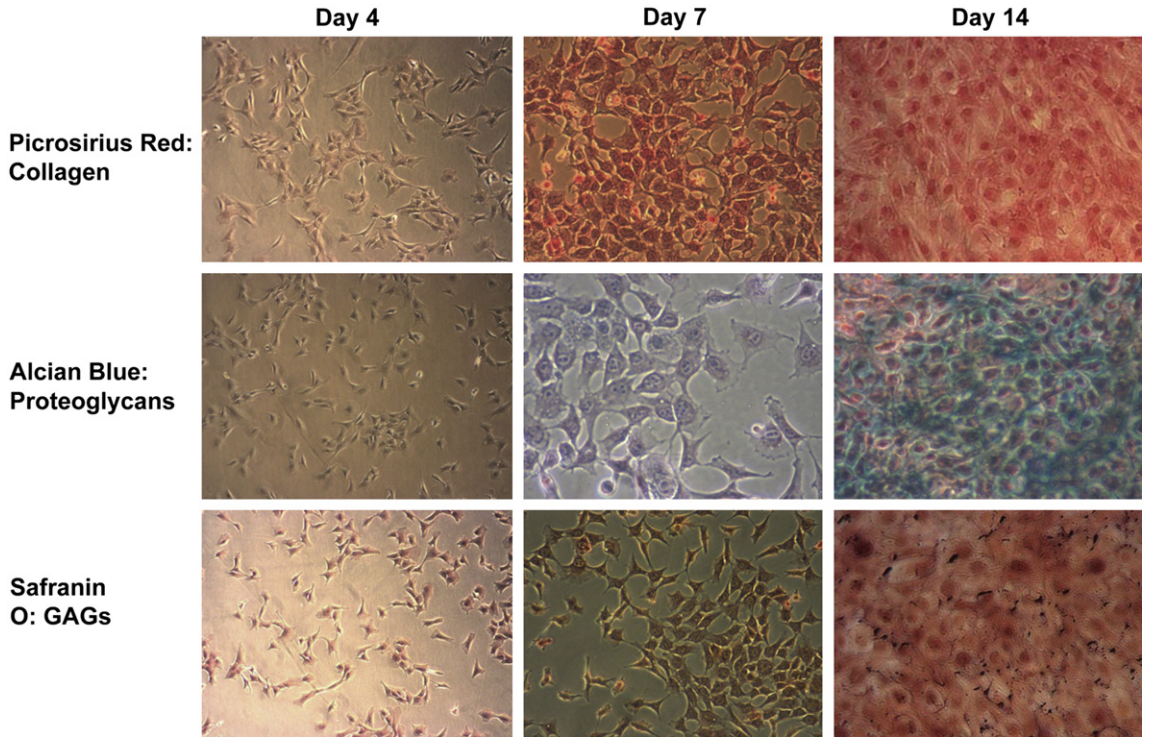

Fig. 5 – Histology staining of ECM proteins, collagenase, proteoglycan, and glycosaminoglycan in chondrocyte cultures at 4, 7, and 14 d. (Color version of figure is available online.)

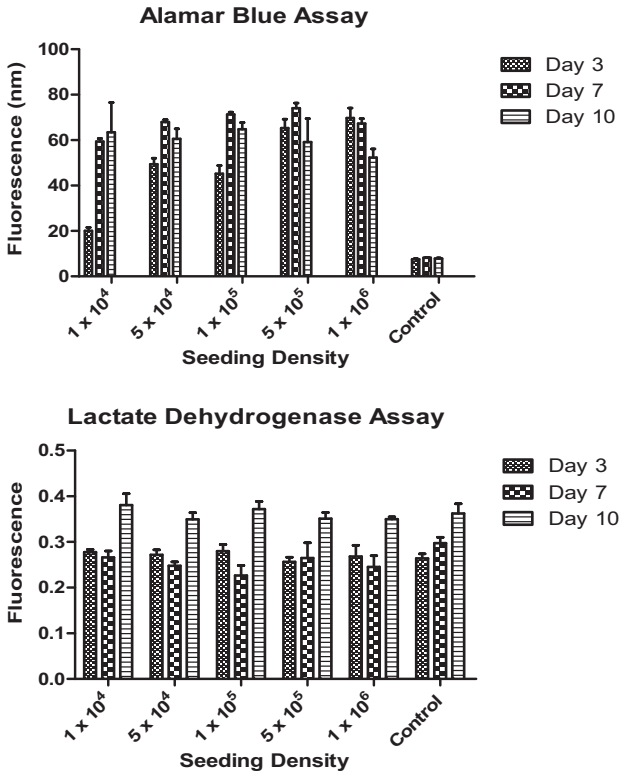

Fig. 6 – alamarBlue and lactate dehydrogenase results for seeding density study.

and temperatures [17]. Cartilage tissue engineering is moving steadily toward widespread use *in vivo*; a number of constructs are implanted into humans, and chondrocytes are used in the therapeutic treatment of osteoarthritis. In light of this, an optimized, validated method for chondrocyte isolation needs to be established and shared with other group. This would serve as a step toward quality assurance. In addition, much of the published literature focuses only on harvesting cartilage from small amounts of cartilage, and therefore protocols rarely work well when scaled up.

In this study, we optimized a protocol for isolating chondrocytes from nasoseptal cartilage that can be scaled up or down to produce consistent results. We first investigated the

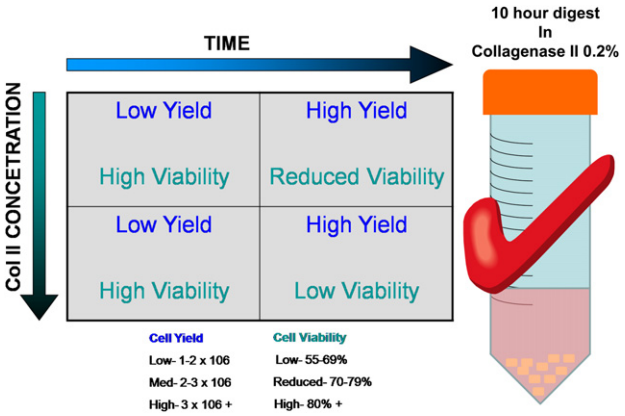

Fig. 7 – Images of optimal digest protocol and effect of time and collagenase concentration on cell yield and viability. (Color version of figure is available online.)

necessity of a predigest phase in chondrocyte isolation. When considering clinical application, simple and short methods are of paramount importance. Our data suggest that the inclusion of a predigest step serves no benefit for increasing the number of viable cells yielded during harvest, and thus is not necessary (Fig. 2).

We evaluated the incubation time and collagenase enzyme concentration in light of previous published research. We found a 10-h digest with an enzyme concentration of 0.2% to be the most effective method for isolating the chondrocytes (Fig. 7). Although increasing the length of incubation time to 16 h dramatically increased the cell yield, the viability was severely reduced. The health of cells in tissue engineering is equally important to the actual number of cells. Interestingly, enzyme concentration did not have as significant an effect on cell viability as the length of incubation time. This is likely the result of a phenomenon observed in our laboratories: If chondrocytes remain freely in suspension for longer than 24 h, they begin to die at fast rate.

We found the optimum seeding density to be 1×105 cells ($P < 0.001$), allowing for a continued growth curve most likely up until 14 d of incubation, enough time for most assays to be conducted. Anything longer than this within a 14-d culture period would lead to exaggerated cell death. For longer periods of culture, the seeding density should be reduced.

The authors have used this protocol in the isolation of human chondrocytes from articular and facial cartilage sources. The results for cell yield and viability are comparable (unpublished data). There is conservation in the types of ECM proteins that are found within the cartilage of the sheep animal model and humans. The nasoseptal cartilage of sheep and humans are both of a hyaline cartilage subtype with a dense collagenase II matrix.

We recommend a 10-h incubation period with 0.2% collagenase II enzyme for optimal chondrocyte yield and viability for cartilage tissue, in which one can expect between 1 and 1.5×106 cells per gram of cartilage. The optimum seeding density on tissue culture plastic 24-well plates is 1×105 for a 10-d incubation. This should be optimized for the different substrates on which the cells are to be grown, because the seeding density is likely to differ.

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Supplementary Data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.jss.2012.05.087.

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