OPTIMIZATION OF PROTOCOL FOR CHONDROCYTE ISOLATION FROM OVINE CARTILAGE

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ABSTRACT

Tissue engineering is one of the most promising alternative therapy for repair of cartilage defects. The large number of cells required for developing cartilage constructs and low cell yield from the cartilage tissue remains the major constraint in cartilage tissue engineering. Hence, an optimised isolation protocol that yields maximum number of viable cells is the first vital step for successful development of cartilage tissue construct. In this study three different isolation protocols using 0.25 % Trypsin-EDTA, 1 % Pronase + 0.4 % Collagenase I and 0.2 % Collagenase II were examined for enzymatic digestion of ovine articular cartilage tissue. Trypan blue staining was used to assess cell yield and viability. Chondrocyte yield per gram was significantly higher ($P < 0.05$) using collagenase II and exhibited better propagation potential when compared to 0.25 % Trypsin-EDTA and 1% Pronase + 0.4 % Collagenase I. Hence, digestion of ovine cartilage using 0.2% collagenase II yielded highest number of viable cells. Further, the isolated cells expressed chondrocyte phenotype and can be used to produce large number of viable ovine chondrocytes.

Key words: Chondrocyte, Collagenase II, Isolation protocol, Ovine cartilage, Trypsin

INTRODUCTION

Cartilage is a poorly cellularized connective tissue with limited intrinsic healing potential. As cartilage is avascular, it lacks the usual inflammatory response of hemorrhage, fibrous clot formation, cellular production and migration of mesenchymal

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cells. Therefore any damage to articular cartilage leads to an incomplete regeneration by local chondrocytes, accompanied with an inferior fibrocartilage formation (McNickle *et al.,* 2008; Camp *et al.,* 2014; Richter *et al.,* 2016). Over the past decade a number of viable options of cartilage regeneration have been introduced into clinical practice with promising results. Optimization of isolation yields, abundant cell sources and efficient culturing procedures that produce desired, reproducible and relatively affordable cell cultures are therefore highly rated novelties in recent research (Gardner *et al.,* 2013; Hildner *et al.,* 2011).

Several methods for chondrocyte isolation from various tissue parts and organisms were introduced (Mirando *et al.,* 2014; Xu and Zhang, 2014). However, tissue engineering consumes many cells as such and chondrocyte yield from cartilage digest is 20 per cent less than the total available cells. Despite this limitation a high cell density is critical for the maintenance of chondrocyte phenotype and matrix synthesis and remains a major challenge in cartilage repair (Schulze-Tanzil *et al.,* 2002). Hence an optimized protocol for isolation forms a powerful tool to boost the yield of chondrocytes in the development of clinical cartilage constructs. This study was therefore taken to optimize protocol for isolation of primary chondrocytes from ovine cartilage for possible tissue engineering.

MATERIALS AND METHODS

Collection of ovine cartilage tissue

The ovine articular joint tissue was collected from the shoulder and knee joints of six apparently healthy young sheep immediately after slaughter irrespective of breed and sex. The samples (n = 6) were collected in phosphate buffered saline containing gentamicin (50µg/ml) and transported to cell culture laboratory under sterile conditions keeping the tissue at 4^0C with the help of ice packs.

Cartilage preparation for digestion

The joint tissue was cleaned with 70 per cent alcohol and rinsed with phosphate buffered saline (PBS) containing antibiotic antimycotic solution. The surrounding muscle tissue was dissected and the joint was disarticulated. The hyaline cartilage encapsulating both the ends of the joint was gently scraped off with the help of a sterile scalpel. The cartilage shavings obtained were then minced with sterile scalpel blade into small pieces (approximately 2 × 2 mm) in a 60 mm culture dish containing DMEM and washed three times with PBS containing 1 per cent antibiotic antimycotic. The minced cartilage tissue, approximately one gram, was subjected to enzymatic digestion.

Isolation of ovine chondrocytes

The media was carefully pipetted out from the minced cartilage and transferred to a sterile petri dish containing 10 ml of digestive enzyme. Isolation of chondrocytes from the ovine cartilage tissue was performed using three different protocols given in Table 1.

Table 1. Enzymes used for chondrocyte isolation at 37°C

S.No.	Enzyme	Concentration (%)	Duration (hours)
1.	**Trypsin EDTA** (Naranda *et al*., 2017)	0.25	3
2.	**a. Predigest: Pronase**	1	1.5
	b. Collagenase type I (Kuettner *et al*., 1982)	0.4	3
3.	**Collagenase type II** (Oseni *et al*., 2013)	0.2	20

After the period of digestion, the cell suspension was filtered through a 70 µm cell strainer into a 50 ml falcon tube and neutralized with equal volume of growth media containing Fetal bovine serum (FBS) for enzyme deactivation. The cells were pelleted by centrifugation at 2500 rpm for 10 mins at 4^0C. The supernatant was carefully discarded and the cell pellet was washed twice with 1X PBS. The cell pellet was then resuspended in 1 ml of growth media containing DMEM, Fetal bovine serum and antibiotic-antimycotic solution. Cell viability was determined by Trypan blue dye exclusion test.

Culture of ovine chondrocytes

Isolated ovine chondrocytes were seeded in T_{25} flasks with a seeding density of 3×10^5 cells per flask. The cells were observed regularly with the microscope and culture media was changed every 3 days.

Subculture of ovine chondrocytes

When the cells reached 80-90 per cent confluence, they were trypsinized and the harvested primary chondrocytes were centrifuged and resuspended in culture medium. The cells were then seeded at density of 3×10^5 cells in T_{25} flask and this was repeated until 3 passages.

Statistical analysis

Data analysis was made using Statistical Package for Social Sciences (IBM SPSS, version 20). One-way Analysis of Variance (ANOVA) was used to analyse the data followed by Duncan's post hoc test. A value of $P < 0.05$ was set as a significant level. Results are expressed as Mean ± SE.

RESULTS AND DISCUSSION

Isolation of ovine chondrocytes

Three different protocols were compared for enzymatic digestion of ovine cartilage. The total and viable yield of ovine chondrocytes using different methods of digestion are presented in Table 2.

Table 2. Chondrocyte yield of different digestion methods per wet gram of cartilage

Enzyme	Total cell yield ($\times 10^6$)	Viable cell count ($\times 10^6$)	Dead cell count ($\times 10^6$)
0.25% Trypsin EDTA	0.93 ± 0.05^a	0.85 ± 0.05^a	0.07 ± 0.01^a
1% Pronase + 0.4 % Collagenase type I	1.23 ± 0.03^b	1.15 ± 0.03^b	0.08 ± 0.01^a
0.2% Collagenase type II	2.75 ± 0.12^c	2.55 ± 0.1^0c	0.19 ± 0.02^b

[a-c] Values within a column with different superscript differ significantly ($p < 0.01$)

Total and viable yield of chondrocytes

Cell viability was confirmed by trypan blue dye exclusion test. The viable cells were observed with intact nucleus and clear cytoplasm without taking the stain particles. The dead cells were stained blue colour with trypan blue dye.

The chondrocyte yield per wet gram of tissue of ovine cartilage digested using 0.2 % Collagenase II was significantly higher ($p < 0.01$) when compared with 0.25 % Trypsin-EDTA and 1 % Pronase + 0.4 % Collagenase I. The viable cell yield after twenty hours of 0.2 % Collagenase II digestion averaged $2.55 \times 10^6 \pm 0.10$. Similar finding was observed by Jakob *et al.,* (2003) in isolation of human articular chondrocytes. Among the latter two methods, 1 % Pronase + 0.4 % Collagenase I yielded significantly higher number of cells ($p < 0.01$) when compared to 0.25 % Trypsin-EDTA.

Naranda *et al.,* (2017) employed 0.25% trypsin EDTA for the isolation of human articular chondrocytes due to its affordability and availability. However, disaggregation using trypsin EDTA can be ineffective for very dense connective tissue such as cartilage as they act on lysine or arginine aminoacid residue on their c-termini unless these amino acids are followed by proline (Olsen *et al.,* 2004).

Several studies have demonstrated the use of Collagenase II in isolation of chondrocytes (Centola *et al.,* 2015; Vedicherla *et al.,* 2017). Since the extracellular matrix of most of the connective tissue is made of collagen, collagenase serves as an effective enzyme for isolating cells from connective tissue like cartilage. Collagen II being structural component of the joint cartilage, unwinds the triple-helical collagen before it cleaves glycine-L-isoleucine and Glycine-leucine peptide bonds (Chung *et al.,* 2006).

A number of protocols for isolation of chondrocytes involving a predigest step using pronase/ hyaluronidase to loosen the tissue for increased enzyme exposure have been reported and hence a predigest step using 1 % Pronase was used in this study. However, predigestion using Pronase did not significantly increase the yield of viable chondrocytes. Similar

finding was observed by Oseni *et al.,* (2013) in the isolation of chondrocytes from ovine nasal septal cartilage.

Morphology of cultured Chondrocytes

The morphology of ovine chondrocytes at different passage levels (P1-P3) isolated using 0.2 % Collagenase II are given in Fig. The morphology of primary chondrocytes isolated using Collagenase II displayed a uniform morphology with good attachment to the culture flask. Most of the cells examined exhibited identical size with polygonal morphology. Cell multiplication was obvious from day 3 and the cells exhibited short cytoplasmic processes with eccentrally placed nucleus. The cells attained 80 % confluency on day 14 where a monolayer of cells with cobblestone appearance was observed.

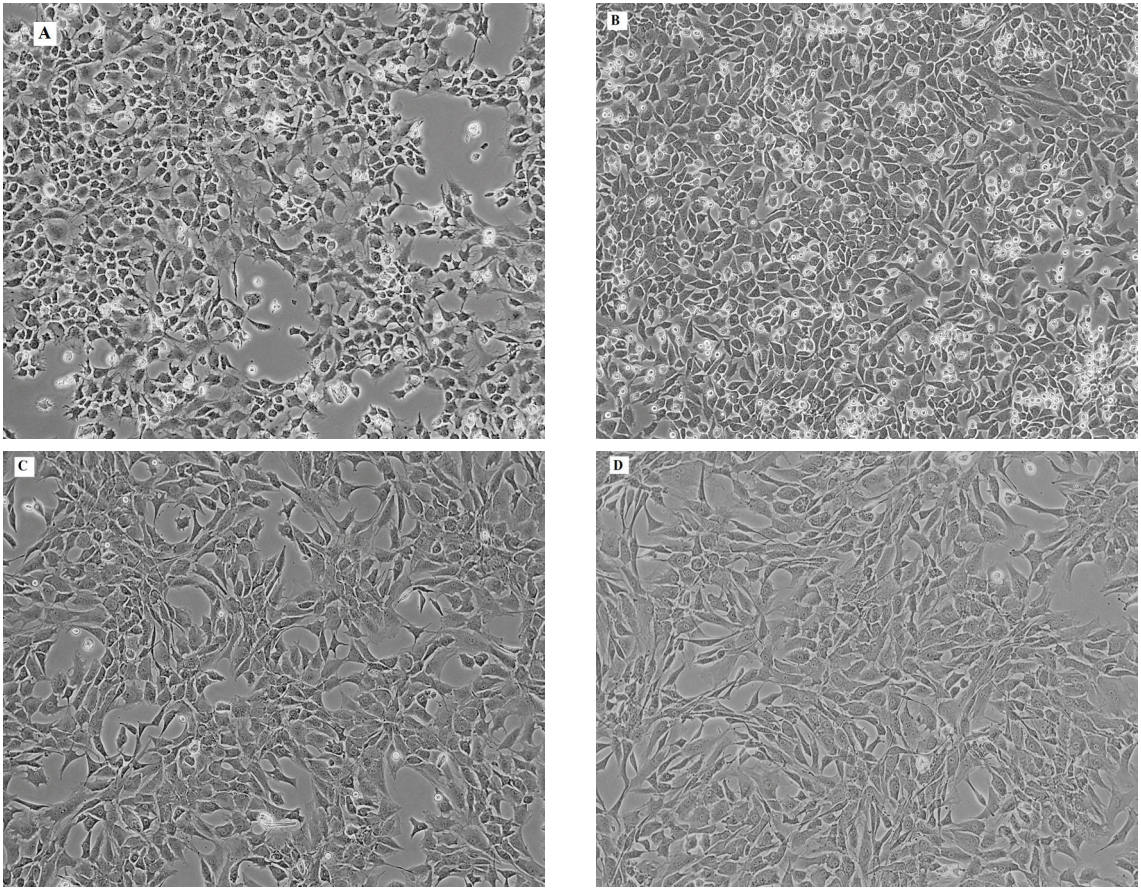

Fig. A: Morphology of primary chondrocytes isolated using 0.2 % collagenase II at day 12 displaying polygonal shape (100x). B,C,D: Chondrocytes isolated with collagenase II at passage 1, 2 and 3 respectively in day 7 with 80 % confluency showing cobblestone morphology (100x).

The primary chondrocytes isolated using 0.25 % Trypsin EDTA and 1 % Pronase + 0.4 % collagenase I showed poor adherence and could not be propagated beyond day 6. The low number of cells obtained during primary expansion hindered subsequent experiment using cells isolated using the latter methods. The low level of attachment exhibited by trypsin and collagenase I might be attributed due to their interference with membrane adhesive proteins such as cadherins and integrins that links cytoskeleton with the extracellular matrix (ECM) (Huang *et al.,* 2010)

CONCLUSION

In the present study different protocols for the isolation of ovine chondrocytes from articular cartilage were compared. Digestion using 0.2 % collagenase II had shown to produce significant number of cells that retained their phenotype with good propagation potential. Hence this protocol can be employed to generate large number of chondrocytes for cartilage based tissue engineering.

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