



## Original Article

## Optimal conditions of collagenase treatment for isolation of articular chondrocytes from aged human tissues

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## ABSTRACT

**Introduction:** There are various types of cartilage, including the auricular and articular cartilages. These cartilages have different functions, and their matrix volume and density of chondrocytes may differ. Thus, different protocols may be required to digest different types of cartilage.

**Methods:** In this study, we examined protocols for the digestion of articular and auricular cartilages and determined the optimal conditions for articular cartilage digestion.

**Results:** Our histological findings showed that the articular cartilage has a larger matrix area and fewer cells than the auricular cartilage. In 1-mm<sup>2</sup> areas of articular and auricular cartilages, the average numbers of cells were 44 and 380, respectively, and the average matrix areas were 0.94 and 0.77 mm<sup>2</sup>, respectively. The maximum numbers of viable cells (approximately  $1 \times 10^5$  cells/0.1 g of tissue) were obtained after digestion in 0.15, 0.3, or 0.6% collagenase for 24 h, in 1.2% collagenase for 6 h, or in 2.4% collagenase for 4 h. In tissues incubated in 0.15 or 0.3% collagenase, the cell numbers were lower than  $1 \times 10^5$ , even at 24 h, possibly reflecting incomplete digestion of cartilage. No significant differences were observed in the results of apoptosis assays for all collagenase exposure times and concentrations. However, cell damage appeared to be greater when collagenase concentrations were high. When cells obtained after digestion with different concentrations of collagenase were seeded at a density of 3000 cells/cm<sup>2</sup>, they yielded the maximum cell numbers after 1 week.

**Conclusions:** We recommend a 24-h incubation in 0.6% collagenase as the optimal condition for chondrocyte isolation from articular cartilage. Moreover, we found that the optimum cell-seeding density is approximately 3000 cells/cm<sup>2</sup>. Conditions determined in this study would maximize the yield of isolated articular chondrocytes and enable the generation of a large quantity of cultured cells.

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## 1. Introduction

Collagenase digestion is the first step in cartilage tissue engineering [1–11]. Incomplete digestion reduces the efficiency of tissue engineering and affects the safety of the process and stability of grafts. Therefore, complete digestion is an important process in cartilage tissue engineering. We believe that complete digestion can be achieved by varying the digestion protocol according to the type of cartilage matrix. In a previous study, we optimized the conditions for isolating and seeding human chondrocytes from the auricular cartilage [1]. Specifically, we showed that 24 h of incubation in 0.3% collagenase or 6 h of incubation in 0.6% collagenase were optimal conditions for isolating chondrocytes from cartilage fragments ranging in size from 250 to 1000 μm. We also showed

that the cell seeding density should be in the range of 3000–10,000 cells/cm<sup>2</sup>. However, other reports described widely different collagenase concentrations (range, 0.04–0.25%; <960 U/mL) and incubation times (range, 4–24 h) for the isolation of human articular chondrocytes [12–16]. There are various types of cartilage besides the auricular cartilage, including the articular cartilage, and each type has a different function [17–20]. Therefore, the volume of the cartilage matrix and density of chondrocytes may differ among cartilage types.

These differences in the matrix necessitate the use of different protocols for digesting different cartilage types, such as the articular and auricular cartilages. In this study, we compared the two aforementioned types of cartilage, which have different density, matrix types, and chondrocytes. We determined the optimal conditions for digesting articular cartilage, following a protocol that we established previously for auricular cartilage digestion, as a reference.

## 2. Methods

### 2.1. Histology

The cartilage was fixed with 4% paraformaldehyde, embedded in optimum cutting temperature (OCT) compound (Sakura, Tokyo, Japan), and cryosectioned into 10- $\mu$ m slices. The sections were stained with Toluidine Blue O. The cell number and matrix area were analyzed using EasyAccess (AD Science Co., Tokyo, Japan).

### 2.2. Chondrocyte isolation

All procedures were approved by the Ethics Committee of the University of Tokyo Hospital (ethics permission number 622). Remnant articular cartilages from three patients with osteoarthritis were obtained during surgery. Sampling was performed in accordance with the principles of the Declaration of Helsinki. The three patients were women aged 74–84 years (mean age, 80 years). The cartilage tissues were thoroughly minced with scissors and tweezers into fragments of 250–1000  $\mu$ m in size [1]. Five different concentrations (0.15, 0.3, 0.6, 1.2, and 2.4%) of collagenase from *Clostridium histolyticum* (product catalog number: 038-10531; Wako Pure Chemical Industries, Osaka, Japan) were used. Collagenase solution from a single lot was used throughout the study (291 U/mL). Since this was a typical lot, no information regarding other types of collagenase was provided by the manufacturer. Approximately 3 mL of the collagenase solution was transferred into a 5-mL tube (BD Falcon, Bedford, MA, USA), and 20 tubes were prepared (four tubes for each concentration). A cartilage fragment weighing approximately 0.1 g was added into each tube, and the tubes were incubated in a 37 °C water bath, with agitation at 150 cycles/min. For each collagenase concentration, we measured the total number of cells, number of viable cells, as well as cell viability, using a NucleoCounter (ChemoMetec, Allerød, Denmark) after 2, 4, 6, and 24 h [1].

### 2.3. Chondrocyte culture

Viable cells were seeded into 6.4-mm plastic culture dishes (96-well plate) coated with collagen type 1. Cells were seeded at densities of 30,000, 10,000, 3000, 1000, 300, and 100 cells/cm<sup>2</sup>, and the optimal cell-seeding density for primary cultures was determined. For apoptosis analysis, cells digested under specific conditions were cultured in 35-mm plastic culture dishes coated with collagen type 1. The culture medium was Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (Sigma Chemical Co., St. Louis, MO, USA) containing 5% human serum (Sigma Chemical Co.), 100 ng/mL

fibroblast growth factor-2 (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan), and 5  $\mu$ g/mL insulin (MP Biomedicals, Irvine, CA, USA) [21].

### 2.4. Photometric analysis of ssDNA in apoptotic cells by enzyme-linked immunosorbent assay (ELISA)

We transferred 5000 cells into each well of a 96-well microplate, and the microplate was centrifuged at 200 g for 5 min. The medium was removed, and 200  $\mu$ L of fixative (80% methanol in phosphate-buffered saline) was added into each well. The microplate was incubated at room temperature for 30 min, and the fixative was then removed. Subsequently, the microplate was incubated at room temperature for 1–2 h to allow for cell attachment to the plate. Cell apoptosis was determined using the ssDNA apoptosis ELISA Kit (CHEMICON® International Inc., Billerica, MA, USA), according to the manufacturer's instructions. Absorbance was measured at 405 nm, using a standard microplate reader (ARVO SX 1420 Multilabel Counter; Perkin Elmer, Waltham, MA, USA).

### 2.5. Statistics

Data obtained from three replicate samples per group were analyzed by t-tests in MS Excel (Microsoft Co., Bellevue, WA, USA). The results are expressed as means  $\pm$  standard deviation.

## 3. Results

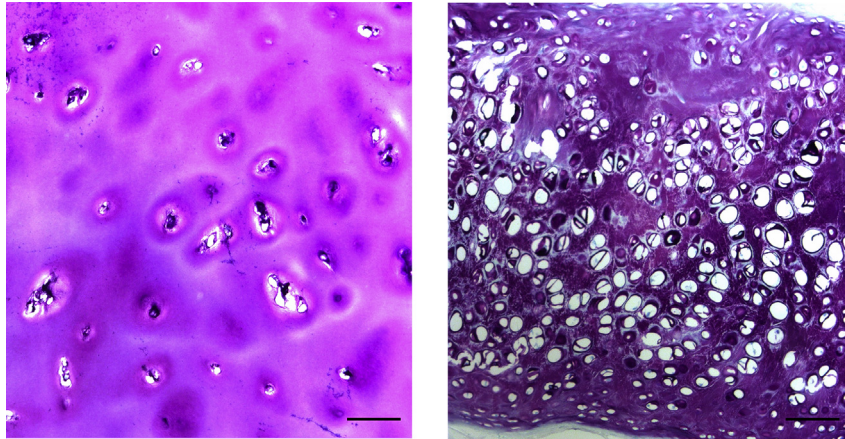
### 3.1. Histology

Histological examination revealed a larger matrix area and fewer numbers of cells in the articular cartilage than in the auricular cartilage (Fig. 1). The average number of cells in a 1-mm<sup>2</sup> area of articular and auricular cartilages was 44 and 380 cells, respectively; the average matrix area in a 1-mm<sup>2</sup> area of articular and auricular cartilages was 0.94 and 0.77 mm<sup>2</sup>, respectively (Fig. 2).

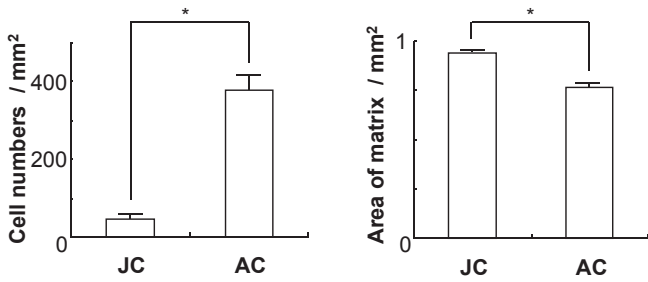
### 3.2. Cartilage digestion

We examined the effects of collagenase concentration and incubation time on cartilage digestion. Cartilage fragments were not completely digested after 24 h of incubation in 0.15 or 0.3% collagenase; however, the fragments were digested after incubation for the same duration in 0.6% or higher concentrations of collagenase (Table 1). The times required for the digestion of cartilage fragments were 6 h and 4 h in 1.2% and 2.4% collagenase, respectively (Table 1).

For all collagenase concentrations, except for the 24 h incubation with 2.4% collagenase, both the total number of cells and the number of viable cells appeared to increase with incubation time (Fig. 3A and B). The number of viable cells from approximately 0.1 g of tissue was close to the maximum number ( $1 \times 10^5$  cells) following incubation in 2.4, 1.2, and 0.6% collagenase at 4, 6, and 24 h, respectively. However, tissues incubated in 0.3% and 0.15% collagenase yielded less than  $8.4 \times 10^4$  cells and  $6.3 \times 10^4$  cells, respectively, even after 24 h (Fig. 3A and B). In other words, the yields were less than those obtained by digestion in 0.6, 1.2, and 2.4% collagenase, which may be due to the incomplete digestion of cartilage fragments incubated in 0.3% and 0.15% collagenase. The total number of cells and number of viable cells obtained from cartilage fragments incubated in 0.15, 0.3, and 0.6% collagenase for 24 h and in 1.2% collagenase for 6 h were significantly higher than those obtained from cartilage fragments incubated at all four concentrations of collagenase for 2 h (Fig. 3A and B). The total and viable cell counts decreased for tissue digested in 2.4% collagenase after 24 h of incubation as a result of reduced viability (Fig. 3C).



**Fig. 1.** Representative histological sections of articular cartilage (left) and auricular cartilage (right) stained with Toluidine Blue O. Thickness of sections, 10 µm; magnification, ×100; scale bars, 100 µm.



**Fig. 2.** The average number of cells (left) and matrix area (right) in a 1 mm<sup>2</sup> area of articular cartilage (JC) and auricular cartilage (AC). \*, P < 0.05.

**Table 1**

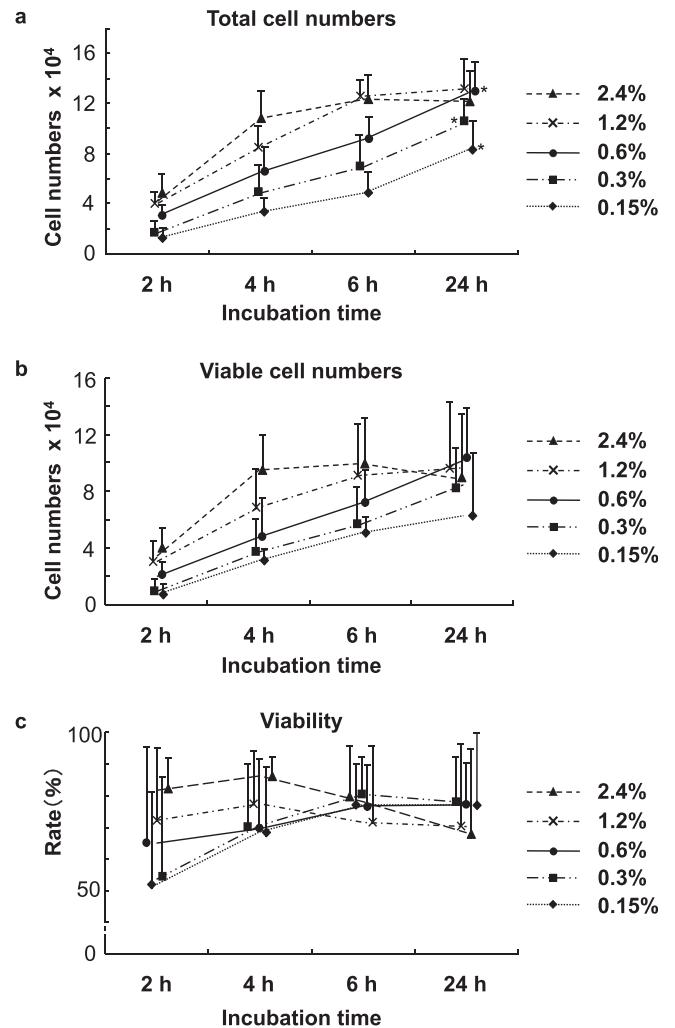
Macroscopic evaluation of collagenase digestion of cartilage fragments. +, many fragments were clearly visible; ±, some fragments were visible; −, all fragments were digested.

	0.15%	0.3%	0.6%	1.2%	2.4%
2 h	+	+	+	±	±
4 h	+	+	+	±	−
6 h	+	+	±	−	−
24 h	+	±	−	−	−

Therefore, to maximize the number of viable cells, the incubation time for cartilage digestion should be 24 h for 0.15, 0.3, and 0.6% collagenase, 6 h for 1.2% collagenase, and 4 h for 2.4% collagenase.

### 3.3. Cell numbers

We also investigated the effect of cell-seeding density on cell growth. Chondrocytes were digested using collagenase under the above-mentioned optimal conditions and were seeded at several densities (100–30,000 cells/cm<sup>2</sup>). Dishes seeded with 3000 cells/cm<sup>2</sup> of cells showed almost confluent growth in 1 week (Table 2). Cells aggregated and formed clusters when chondrocytes from tissues digested in 1.2% and 2.4% collagenase were seeded at 30,000 cells/cm<sup>2</sup> (Table 2). We found that when cells obtained after tissue digestion in 0.15, 0.3, or 0.6% collagenase for 24 h, in 1.2% collagenase for 6 h, or in 2.4% collagenase for 4 h were seeded at a density of 3000 cells/cm<sup>2</sup>, maximum cell numbers were achieved after 1 week (Fig. 4). This finding supported the results shown in Table 1. However, for cells seeded at less than 1000 cells/cm<sup>2</sup>, confluent growth was observed after more than 2 weeks.



**Fig. 3.** Cell numbers and viability after collagenase digestion. Total (a) and viable (b) cell numbers. (c) Cell viabilities. Data from three samples per group are presented as means and standard deviations. Statistical analyses were performed using Dunnett's tests (\*P < 0.05, as compared to the 2-h incubation for each concentration of collagenase).

**Table 2**  
The effect of cell-seeding density on cell growth. The table shows the degree of cell growth. ○, Confluent growth; △, did not reach confluence; ×, aggregation.

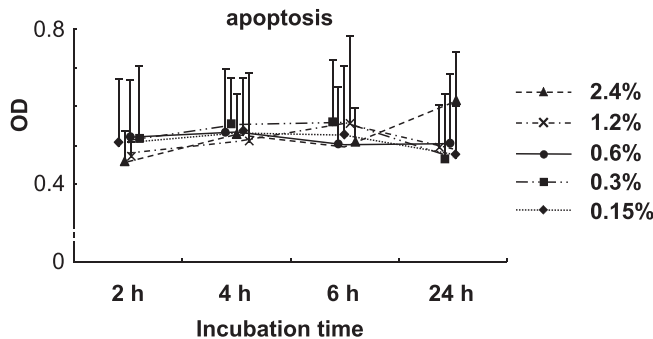
	0.15%/24 h	0.3%/24 h	0.6%/24 h	1.2%/6 h	2.4%/4 h
30,000	○	○	○	×	×
10,000	○	○	○	○	△
3000	○	○	○	○	○
1000	△	△	△	△	△
300	△	△	△	△	△
100	△	△	△	△	△

Results from the apoptosis assay showed that different exposure times and collagenase concentrations did not cause significant differences in cell apoptosis (Fig. 5).

**4. Discussion**

Articular cartilage is a subtype of hyaline cartilage, and auricular cartilage is a subtype of elastic cartilage. The matrix of elastic cartilage is similar to that of hyaline cartilage, but elastic cartilage has characteristic elastic fibers and elastic lamellae. In elastic cartilage, many chondrocyte nuclei are seen. Histological examinations have shown that the number of cells per unit area in the articular cartilage is approximately one-ninth of that found in the auricular cartilage. In addition, the number of cells per unit weight of the articular cartilage is approximately one-ninth of that in the auricular cartilage; these findings are similar to those of our histological examination. Chondrocytes are approximately 50 μm in diameter. In this study, the thickness of the sections was 10 μm and we counted the cells on the histological thin sections. We visualized the chondrocytes by transmitted light microscopy, and cells within the volume of the thin section, rather than just those on the plane of the thin section, were enumerated. Therefore, we believe that the ratio of the cell number (as counted by the histological examination) in the articular versus auricular cartilage was equivalent to that per unit weight. These findings indicated that a modified protocol was necessary. Previous histological and biochemical analyses of the articular cartilage cellularity using specimens similar to those used in this study yielded similar results [22]. However, other studies warned that the density of chondrocytes can vary between healthy and diseased cartilage specimens, as well as with tissue location, depth, and maturity [23,24].

As stated above, the articular cartilage belongs to the hyaline cartilage type. In our study, only cartilages that were not calcified were dissected and harvested. The dissected tissues contained minimal to no bones and synovium. Therefore, cells harvested from the tissue were nearly homogenous and can be classified as chondrocytes. However, after serial passaging of the cultured cells, the properties of the chondrocytes changed into those of dedifferentiated cells. To minimize the number of passages and avoid dedifferentiation, the study was aimed to optimize the number of cells harvested.

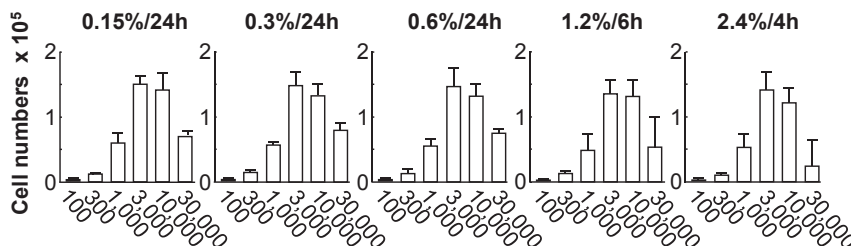


**Fig. 5.** Analysis of apoptosis by ELISA. Data from three samples per group are presented as means and standard deviations. Statistics were assessed using the Dunnett's test.

In primary culture, the maximum number of cells obtained from 0.1 g of articular cartilage after 1 week ( $1.5 \times 10^5$ ) was less than that obtained from 0.1 g of auricular cartilage after the same culture duration ( $6.0 \times 10^5$ ) [3]. A previous study that compared chondrocytes from different cartilage tissues [17,25–30] found that the proliferative abilities of same-aged articular and auricular cartilages were different after 3 weeks of culture; however, no difference in the proliferative abilities of same-aged articular and auricular cartilages was observed after a 1-week culture period in one study [17]. In addition, Tay et al. observed no significant difference between the growth rates of these subtypes of cartilage in humans [25]. However, the proliferative ability of chondrocytes has been shown to decrease with age [26,27]. Therefore, age is considered the main factor influencing the proliferative ability of chondrocytes. In this study, articular cartilages were isolated from old individuals, and these cartilages had more matrix area and fewer cells than the auricular cartilage isolated from younger individuals in a previous study. Consequently, culture time should be increased according to tissue characteristics. This study used a protocol appropriate for cartilages from older individuals.

We counted the cells for tissues incubated in various concentrations of collagenase at various time intervals. The maximum number of viable cells ( $1 \times 10^5$  cells) was harvested from 0.1 g of tissue incubated in 0.6, 1.2, and 2.4% collagenase for 24, 6, and 4 h, respectively (Fig. 3B). The cell numbers for tissues incubated in 0.15% and 0.3% collagenase were lower than  $1 \times 10^5$  cells even at 24 h, possibly because of incomplete digestion of the cartilage. Mincing the tissue can damage the cells located on the surface of the tissues and decrease their viability. Therefore, cells harvested after 2 h of incubation showed lower viability for lower collagenase concentrations (Fig. 3C). The cytotoxic effects of long-term collagenase exposure may have resulted in the low number of viable cells when tissues were incubated in 2.4% collagenase for 24 h.

Furthermore, we showed that a cell seeding density of 30,000 cells/cm<sup>2</sup> may be too high because the cells harvested after



**Fig. 4.** Effect of cell-seeding density on cell numbers after 1 week of culture. Data from three samples per group are presented as means and standard deviations.

**Table 3**  
Comparison of methods and results of cell isolation from the articular and auricular cartilages.

Types of cartilage		Articular cartilage	Auricular cartilage
Subtypes of cartilage		Hyaline cartilage	Elastic cartilage
Conditions	Mean age (years)	80	8
	Size of cartilage fragments ( $\mu\text{m}$ )	250–1000	
Cell Number/ $\text{mm}^2$		44	380
Extracellular matrix area/ $\text{mm}^2$ ( $\text{mm}^2$ )		0.94	0.77
Optimal conditions	Collagenase Concentrations (%)	0.6	0.3–0.6
	Incubation time (hours)	24	6–24
	Cell-seeding density ( $\text{cells}/\text{cm}^2$ )	3000	3000–10,000

seeding (cells from tissues digested in 1.2–2.4% collagenase) at this density tended to aggregate (Fig. 4). We observed no difference in cell growth between chondrocyte culture from the articular cartilage, as performed in this study, and that from the auricular cartilage, which was performed in a previous study (Table 2 and Fig. 4) [1].

As demonstrated by the apoptosis assay, increasing collagenase concentrations did not significantly affect cell death. However, we observed a trend toward increasing cell damage in the presence of high collagenase concentrations, consistent with the results of previous reports [1,22].

## 5. Conclusions

Our results indicated that 0.15% of collagenase is not appropriate to achieve the maximum cell viability. In addition, owing to incomplete digestion, 0.3% collagenase should also be avoided. We recommend 24 h of incubation in 0.6% collagenase as the optimal condition for chondrocyte isolation from articular cartilage fragments ranging from 250 to 1000  $\mu\text{m}$  in size. We found that the optimal cell seeding density was approximately 3000  $\text{cells}/\text{cm}^2$  (Table 3). In our study, 0.6% collagenase was equivalent to 1.7 U/mL of collagenase. The activity of collagenase, which may vary depending on the collagenase source and lot, will need to be considered. The protocol should be optimized not only in terms of cell numbers, but also with regard to cell growth, energetic state, and distribution in cell cycle stages. However, based on findings of the current study, we recommended a protocol to maximize the harvest of viable cells, which is essential for clinical applications. We conclude that the incubation time for digestion of the articular cartilage in 0.6% collagenase has to be increased from 6 h (used for the auricular cartilage) to 24 h because the matrix area for the articular cartilage is larger than that for the auricular cartilage.

## Conflicts of interest

None.

## Acknowledgments

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