Systematic Study of Cell Isolation from Bovine Nucleus Pulposus: Improving Cell Yield and Experiment Reliability

Juliana T. Y. Lee, Kenneth M. C. Cheung, Victor Y. L. Leung

Department of Orthopaedics and Traumatology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Pokfulam, Hong Kong SAR, China

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ABSTRACT: Differences in matrix compositions in human nucleus pulposus (NP) clinical samples demand different cell isolation protocols for optimal results but there is no clear guide about this to date. Sub-optimal protocols may result in low cell yield, limited reliability of results or even failure of experiments. Cell yield, viability and attachment of cells isolated from bovine NP tissue with different protocols were estimated by cell counting, Trypan blue staining and cell culturing respectively. RNA was extracted from isolated cells and quantified by Nanodrop spectrometry and RT-qPCR. Higher collagenase concentration, longer digestion duration and pronase pre-treatment increased the cell yield. Cell viability remained high (<5% dead cells) even after 0.2% collagenase treatment for overnight. NP cells remained to have high *ACAN*, *COL2A1*, *CDH2*, *KRT18*, and *KRT19* expression compared to muscle cells for different cell isolation conditions tested. Digestion by collagenase alone without the use of pronase could isolate cells from human degenerated NP tissue but clusters of cells were observed. We suggest the use of the disappearance of tissue as an indirect measure of cells released. This study provides a guide for researchers to decide the parameters involved in NP cell isolation for optimal outcome.
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Intervertebral disc (IVD) is an important part of the spine and its degeneration is highly associated with back pain, which is one of the major health problems in many developed countries.[1–5] The major components of IVD are nucleus pulposus (NP), annulus fibrosis (AF) and cartilaginous endplates (CEP).[6] To elucidate the mechanism related to disc degeneration, primary NP cells were isolated from the NP tissue of different animal species for different in vitro studies. The cell isolation from tissues is often achieved by enzymatic digestion using collagenase. There are various protocols for NP cell isolation and they vary greatly in terms of enzyme concentrations (0.01–0.5%), digestion duration (30 min to overnight) and type of pre-collagenase treatments such as digestion by pronase, which is a mixture of different proteases (Table 1). To date, there is no systematic study about the cell isolation protocols for NP tissues and sub-optimal protocols may result in lower cell yield, which can be a critical problem for human IVD samples as their availability are usually more limited. Human NP was suggested to have most dramatic changes with age of any cartilaginous tissue[7] and variability was observed in clinical samples obtained from patients of different ages with discs of different degrees of degeneration. Theoretically in scientific studies, the same protocol should be used throughout the same set of experiments but for clinical NP samples, using the same protocol does not guarantee similar isolation efficiency as the samples themselves can be quite different. Interestingly, the digestion duration of human NP in reported studies can be as short as 1 h[8] but also as long as overnight.[9] When the tissues were insufficiently digested, the cells in the tissues could not be released and sufficient RNA could not be extracted for reliable gene expression studies. This necessitates better understanding of the cell isolation process.

Among different species, we chose bovine NP tissue for studying various factors affecting the cell isolation procedures since bovine IVD has higher similarity to human IVD in terms of cell distribution, cell phenotype, disc composition, disc size, and mechanical loading. Unlike rodent NP cells which are in direct cell-cell contact, cells in bovine caudal NP tissues are at low cell density and are separated by extracellular matrix. Cows and sheep resemble human in that they may have some notochordal cells at birth but the numbers decrease rapidly with age, while most other species such as mouse, rat, cat, mink, dog, pig, and rabbit all have notochordal cells in the NP at birth and retain them throughout much of their adult life.[10] Besides, bovine coccygeal discs have similar NP composition compared with human 40–80 years of age in terms of water content (77%), proteoglycan content and type II collagen content.[11] Bovine caudal discs (14–22 mm in diameter and 5–10 mm thick) are comparatively close in size to the human lumbar discs and the musculature of the bovine tail maintains an in vivo pressure on the discs that is approximately the same as in the human lumbar discs in the prone position (0.1–0.3 MPa).[10] In addition, Oshima et al. reported that the swelling pressure in bovine tail discs is similar to that in human lumbar discs, indicating

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Correspondence to: Juliana T. Y. Lee (T: +852 6181 2078; F: +852 2818 5210; E-mail: julee@connect.hku.hk)
Co-correspondence: Kenneth M. C. Cheung (cheungmc@hku.hk); Victor Y. L. Leung (vicleung@hku.hk)

Table 1. Summary of Cell Isolation Conditions and Durations for IVD Tissues in Some Reported Studies

Reference	Pre-Collagenase Treatment	Collagenase Treatment[a]	Tissue[b]	Species	Notes
Minogue et al. 2010[26]	0.5% protease for 1 h	0.5% collagenase II + 0.1% hyaluronidase for 2–3 h	NP, AF	bovine	serum free medium
Bron et al. 2011[27]	2.5% protnase E for 1 h	0.125% collagenase for 16 h	NP, AF	goat	1%, 25% FBS
Gilbertson et al. 2008[28]	0.2% pronase for 90 min	0.02% collagenase II for O/N	NP, AF	human	with gentle agitation
Risbud et al. 2006[29]		0.1% collagenase + 10 U/ml hyaluronidase for 4-6 h	NP, AF	rat	
GantenbeinRitter et al. 2012[30]	0.19% pronase for 1 h	collagenase II for 14 h	NP	bovine	1-year-old
GantenbeinRitter et al. 2011[31]	0.19% pronase for 1 h	0.4% collagenase II for O/N	NP	bovine	6- to 12-month-old
Arana et al. 2010[32]	0.5% protease for 1 h	0.1% collagenase A for O/N	NP	bovine	
Gilson et al. 2010[33]		0.05% type I collagenase for O/N	NP	bovine	
Halloran et al. 2008[34]	0.2% pronase + 0.004% DNase II for 90 min	0.05% collagenase II + 0.01% hyaluronidase + 0.004% DNase II for O/N	NP	bovine	
Jones et al. 2008[35]		0.8 mg/ml crude type XI collagenase + 1.67 U/ml DNase for O/N	NP	bovine	18- to 32-month-old
Korecki et al. 2009[36]	0.2% pronase for 1 h	0.125% collagenase type IV for 8–10 h	NP	bovine	constant agitation
Zeiter et al. 2009[37]	0.19% pronase for 1 h	32 IU/ml collagenase II for 10 h	NP	bovine	
Watanabe et al. 2010[38]	0.27% pronase for 1 h	0.025% collagenase for 2 h	NP	human	
Yang et al. 2010[9]		0.2% collagenase for O/N	NP	human	
Purmessur et al. 2008[39]	2 U/ml protease for 30 min	0.4 mg/ml collagenase I for 4 h	NP	human	
Studer et al. 2007[40]	0.2% pronase for 90 min	0.02% collagenase type II for O/N	NP	human	
LeMaitre et al. 2005[41]	2 U/ml protease for 30 min	0.4 mg/ml collagenase I for 4 h	NP	human	
Fernando et al. 2011[42]		0.75 mg/ml collagenase type II + 0.6 mg/ml protease for O/N	NP	pig	
Guehring et al. 2009[43]	0.2% protease for 1 h	0.2% collagenase for 18 h	NP	pig	
Cheng et al. 2011[44]		0.2% collagenase for 18 h	NP	rabbit	10% FBS
Yuan et al. 2011[45]	0.2% pronase for 1.5 h	0.05% collagenase IA for 16 h	NP	rabbit	
Su et al. 2010[46]		0.01% collagenase for 16 h	NP	rabbit	
Hiyama et al. 2011[14]	0.01% trypsin for 15 min		NP	rat	
Hiyama et al. 2010[47]		0.0125% collagenase P + 0.4% pronase for 30 min	NP	rat	
Kakutani K 2006[8]		0.25% collagenase for 1 h	NP	rat, human	
GantenbeinRitter et al. 2012[30]	0.19% pronase for 1 h	collagenase type 2 for 14 h	NC	pig	4- to 5-month-old
Guehring et al. 2009[43]	0.2% protease for 1 h	0.025% collagenase for 18 h	NC	pig	Additional 2 h digestion in nonenzymatic cell dissociation solution

Table 1. Continued

Reference	Pre-Collagenase Treatment	Collagenase Treatment[a]	Tissue[b]	Species	Notes
Gilson et al. 2010[33]		0.075% type I collagenase for O/N	AF	bovine	
Korecki et al. 2009[36]	0.2% pronase for 1 h	0.2% collagenase type IV for 8–10 h	AF	bovine	with constant agitation
Gilbert et al. 2010[48]	300–350 PUK/ml pronase for 1 h	0.25% collagenase II + 0.01% hyaluronidase for 4 h	AF	human	
LeMaitre et al. 2005[41]	2 U/ml protease for 30 min	2 mg/ml collagenase I for 4 h	AF	human	
Fernando et al. 2011[42]		1.5 mg/ml collagenase type II + 0.6 mg/ml protease for O/N	AF	pig	
Guehring et al. 2009[43]	0.2% protease for 1 h	0.6% collagenase for 18 h	AF	pig	
Hiyama et al. 2010, 2011[14; 47]	0.4% pronase for 1 h	0.025% collagenase P for 3 h	AF	rat	
Wan et al. 2007[49]		0.1% collagenase II for 3–6 h	AF	rat	

[a]Collagenase preparation from different companies may have different classifications of collagenase types.[b]NP, nucleus pulposus; AF, annulus fibrosus; NC, immature nucleus pulposus with notochordal cells.

that the prevailing compressive stress of the tail discs is probably of similar magnitude to lumbar discs.[12] Thus bovine disc cells may be more clinically relevant to the study of IVD degeneration.

In this optimization study, we started with characterizing the bovine tissues to provide information in designing our experiments. Then we focused on three main applications of the isolated NP cells, namely for cell culturing, RT-qPCR, and flow cytometry.

MATERIALS AND METHODS

Characterization of Bovine IVD Tissues and Cells

The details of the methods in tissue and cell characterization are given in the supplementary material. In brief, the viability of cells in bovine NP and AF tissues was assessed using LIVE/DEAD® stain (Invitrogen, cat # L3224). The water content was estimated by weighing the tissues before and after freeze-drying and the proteoglycan contents were estimated by 1,9-dimethylmethylene blue (DMMB) assays. The cell diameters were measured from the phase contrast photos of isolated cells. The cell densities in NP and AF tissues were obtained by counting the numbers of cells in photos of hematoxylin-stained cryosections. For NP tissue, the cell density was also estimated from cell isolation experiments.

Cell Yield, Cell Viability, and Attachment of Isolated Cells

Two percent pronase stock solution and 5% collagenase stock solution were prepared by dissolving the lyophilized pronase (Roche, cat # 11 459 643 001) and Type 2 collagenase (Worthington) respectively in water and filtered using 0.22 μm syringe filters (Millipore). The enzyme stocks were diluted to the desired concentrations using high glucose Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, cat # 12100-046) with N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Sigma, cat H4034) added. Fetal bovine serum (FBS, Biosera, FB-1001/500) was added to final concentrations of 10%. Antibiotics (penicillin and streptomycin, Gibco, cat # 15140) and fungizone amphotencin B (Invitrogen, product # 15290-018) were added to medium to a final concentration of 100 U/ml, 100 and 1 μg/ml respectively. Prior to cell isolation, NP tissues were cut into 2 mm pieces using a scalpel. The pieces of NP were mixed and distributed evenly into plastic tubes and different enzymes were added and incubated with conditions as shown in Figure 2 in the main text (0.2% pronase, 0.25% Trypsin, 0.1% dispase). After the digestion, the cell suspensions with undigested tissues were filtered through small tailor made metal strainers made from QIAshredder (Qiagen) and metal sieves with pores of 23 μm to remove undigested tissues. The cell suspension was centrifuged and most supernatant was removed leaving 100 μl of medium behind. The cell pellets were resuspended in the supernatant and counted using a hemacytometer after mixing with 0.4% Trypan blue (Sigma, cat # T8154) in 1:1 ratio. For cell attachment assessment, the cells without being Trypan blue stained were washed with phosphate buffered saline (PBS) for three times and re-suspended in DMEM without HEPES. The cells were plated in a 48-well plate and photos were taken at 0, 1, 4, and 7 days after the harvest of tissues.

For isolation of cells from rat NP tissue, the NP was digested with 0.2% pronase for 1 h with subsequent 2 h collagenase treatment or 3 h collagenase treatment without pronase pre-treatment. Shorter digestion time in collagenase was used for rat NP compared to bovine NP as the rat NP tissue is smaller and the digestion time used in literatures is in general shorter for rat NP than bovine NP (ranges from 30 min to 6 h for rat NP and 2 h to overnight for bovine NP) (Table 1).

Quality of RNA Extracted With Different Digestion Conditions

For RNA extraction, higher concentrations of collagenase (1 and 2%) were used. The collagenase stock solution was prepared in DMEM instead of water to avoid significant dilution effect to the salts present in the DMEM due to stock solution addition. 2% collagenase solution was prepared by dissolving Type 2 collagenase (Worthington) in DMEM with

Table 2. Water, GAG Contents, and Cell Densities in Bovine NP and AF Tissues (Mean ± SD)

	NP	AF
1. Water content (%)	76.9% ± 3.2%	65.4% ± 8.7%
2a. Proteoglycan content (% wet weight)	7.7% ± 1.0%	2.9% ± 1.8%
2b. Proteoglycan content (% dry weight)	33.9% ± 5.2%	9.7% ± 7.4%
3a. Estimated volume per cell (μm^3) (based on micrographs of tissue cryosections)	1252 ± 879[a]	702 ± 285[a]
3b. Planar cell density (per mm^2) (from micrographs of tissue cryosections)	124.2 ± 70.4	483.2 ± 260.9
3c. Volumetric cell density (per mm^3) (based on micrographs of tissue cryosections)	1530 ± 1385	11724 ± 9038
3d. % volume of cells in tissues (based on micrographs of tissue cryosections)	0.19% ± 0.17%	0.82% ± 0.63%
4a. Cell diameter (μm) (based on isolated cells)	13.3 ± 2.3	13.0 ± 2.1
4b. Estimated volume per cell (μm^3) (based on isolated cells)	1340 ± 648	1249 ± 594
4c. Cell density (per mm^3) (from cell isolation)	868.7 ± 117.5	N/A
4d. % volume of cells in tissues (based on cell isolation)	0.11% ± 0.01%	N/A

[a]The volume of cells based on cryosections may be underestimated as explained in the supplementary file.

HEPES and filtered using 0.22 μm syringe filters (Millipore). The required concentrations of the enzyme solutions were prepared by diluting the stock solutions with DMEM with HEPES. Prior to cell isolation, NP, AF and muscle tissues were cut into 2 mm pieces using a scalpel and digested in different conditions as shown in Table 3 in the main text. Then the digested samples were centrifuged and washed with PBS. The cell suspension was centrifuged again and then the supernatant was removed. TRIzol (Invitrogen, cat # 15596-018) was added to the cell pellets to lyse the cells and the lysates were stored in a −80°C freezer prior to RNA extraction. The details of the RNA extraction and RT-qPCR are given in the supplementary file. The yield was estimated based on the corrected RNA concentrations calculated using a formula developed by our group[13] to correct the possible overestimation caused by phenol contamination.

Disappearance of Tissues and Number of Isolated Cells

Bovine NP tissues were digested in 0.2% collagenase in DMEM. Photos of the digestion were captured at 1, 2, and 4 h. At these specific time points, the number of isolated cells and the amount of undigested tissues were measured with the details given in the supplementary file.

Cell Density Estimation of Human IVD Tissues and Cell Isolation

The demographic information of IVD donors in this study was listed in the supplementary file. Paraffin sections (6 μm thick) of human IVD tissues on glass slides were dewaxed, rehydrated, and mounted with VECTORSHIELD DAPI mounting medium. Photos were captured and cell densities were estimated from the photos.

In order to test whether cells can be isolated for cell culture using collagenase alone, human IVD tissues were obtained from a 42-year-old female donor with degenerative disc disease (DDD). The tissues were digested overnight in 0.3% collagenase and the isolated cells were seeded in culture flasks (details given in the supplementary file). IVD from another donor (68-year-old male donor with DDD) was used for cell isolation using different digestion conditions (details given in the supplementary file).

Statistical Analysis

P-values were calculated using Student's two-tailed t-test of unequal variance.

RESULTS

Cell Distribution, Cell Densities, and Tissue Compositions of Bovine NP and AF

Cells in bovine IVD were solitary and separated from each other by the extracellular matrix and higher percentages of dead cells were observed in AF tissue than NP tissue after tissue harvest. Figure 1A–D showed the distribution of cells in NP and AF tissues stained with Invitrogen LIVE/DEAD® stain, which stained living cells green and dead cells red. Most cells in the NP and AF tissues were not in direct cell-cell contact and there were more dead cells in AF tissue compared with NP tissue in general. Figure 1E and F shows cryosectioned tissues stained with hematoxylin and there were more cells in AF than in NP, which is consistent with the tissues stained with LIVE/DEAD® stain.

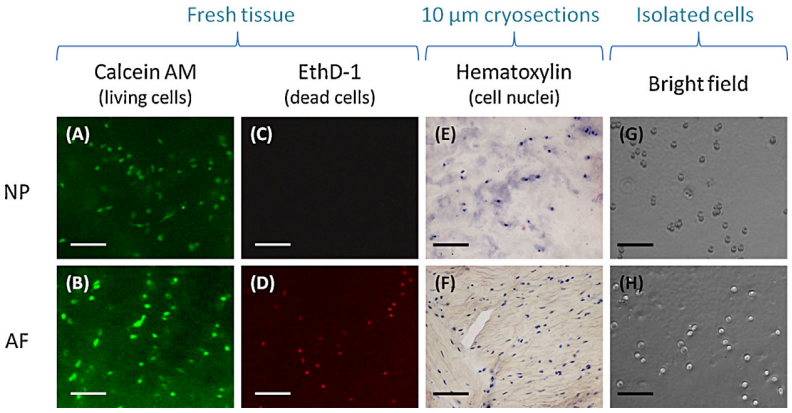


Figure 1. (A–D) Cell distribution in bovine NP and AF tissues revealed by live/ dead staining; (E, F) NP and AF tissues stained with hematoxylin for visualization of cell nuclei; (G, H) NP and AF cells isolated by collagenase digestion (scale bar = 100 μm).

Similar to human IVD, cells only constitute a small volume in the bovine IVD tissues. The volume of cells estimated from tissue sections were 1,252 μm^3 and 702μm^3 for bovine NP and AF respectively. The cell densities estimated from these cryosection photos were 124 cells per mm^2 (1,530 cells per mm^3) and 483 cells per mm^2 (11,700 cells per mm^3) for NP and AF respectively (Table 2). Based on these data, NP cells occupies about 0.19% of NP tissue by volume while AF cells occupies a larger volume of 0.82%. Figure 1G and H shows the cells isolated from bovine NP and AF with collagenase treatment and we also quantified the cell sizes by measuring the cell diameters in photos of isolated cells. On average, the cell diameter of NP is 13.3 μm and that of AF is 13.0 (Table 2). By using this information, the percentage volume occupied by cells in NP tissue based on cell isolation was estimated to be about 0.11%. The water content of NP and AF are 76.9 and 65.4% respectively (Table 2). The bovine NP tissue has higher sulfated glycosaminoglycans (GAG) content (7.7% of wet weight and 33.9% of dry weight) compared to AF tissue (2.9% of wet weight and 9.7% of dry weight).

Cell Yield, Viability, And Attachment

Among the protocols tested, 0.2% collagenase treatment overnight gave the highest yield but also resulted in a higher percentage of cell death. On average, each gram of NP tissue yields 7.2×10^5 cells after complete digestion of the tissue. For low collagenase concentration (0.05%) treatment, a pronase incubation step prior to collagenase treatment gave much higher yield for 6 h of digestion duration (Fig. 2A). More cells could be released after longer time of collagenase incubation. Higher concentration (0.2%) of collagenase yielded a larger cell number when compared with lower concentration (0.05%) after 6 h of digestion (Fig. 2B). After overnight digestion, the tissues disappeared for both 0.05 and 0.2% collagenase concentrations and this gave the highest possible yield available from the tissues.

Most cells remained alive even after the digestion with high collagenase concentration (0.2%) for overnight. In the Trypan blue exclusion staining, only less than 5% of cells were stained blue, indicating most cells are living and the average % of dead cells is lower for shorter digestion duration or lower collagenase concentration (Fig. 2B). Besides, majority of cells were able to attach to the plastic culture plate upon plating after a week (Fig. 3A) and this confirmed the high viability of cells from Trypan blue staining.

The isolated NP cells attached to plastic culture surface and spread like fibroblasts though the speed of attachment was slower than established cultures. The size and shape of the bovine NP cells were similar to the AF cells but slightly different from the muscle cells

Table 3. Yield and Quality of RNA Obtained with Different Isolation Methods

Cells[a]	Yield (μg RNA per g Tissue)[c]	A260/A280	A260/A230
NP col 1% 2 h	4.00 ± 1.38	2.00 ± 0.05	1.32 ± 0.22
NP col 1% 3 h	4.96 ± 1.08	2.00 ± 0.05	1.15 ± 0.23
NP col 2% 2 h	5.41 ± 2.20	2.02 ± 0.04	1.27 ± 0.28
NP col 1% pro 0.1% 3 h	5.81 ± 1.76	2.01 ± 0.04	1.28 ± 0.21
AF col 1% pro 0.1% 6 h[b]	2.78 ± 0.88	2.02 ± 0.06	1.35 ± 0.22
muscle col 1% pro 0.1% 3 h	5.41 ± 3.00	2.06 ± 0.09	1.66 ± 0.30

The quantity of RNA was calculated using the absorbance at 260 nm and the quality was assessed by determining the ratio of the absorbance at 260 nm to the absorbance at 280 nm and the ratio of the absorbance at 260 nm to the absorbance at 230 nm.
[a]"col" denotes "collagenase", "pro" denotes "pronase."[b]6 h of digestion of AF was used instead of 3 h since the numbers of collected cells with 3 h of digestion was not enough for RNA extraction and a large proportion of AF tissue was not completely digested.[c]The yield of RNA was calculated based on concentrations corrected with a mathematical formula[13] (mean ± SD; $n = 6$; from two batches of bovine tails).

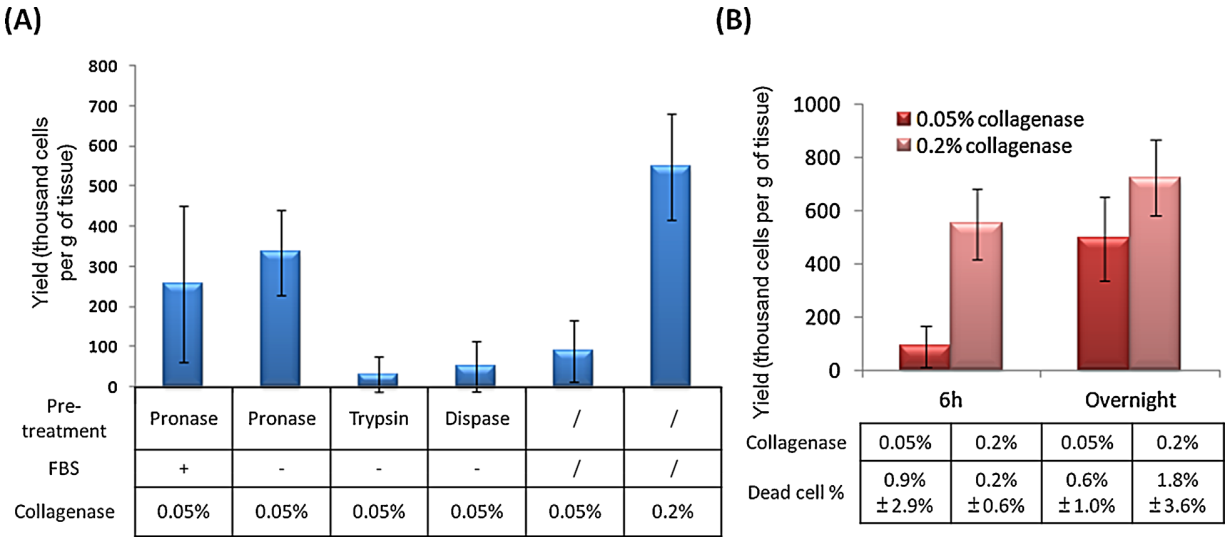


Figure 2. (A) Effect of different pre-collagenase treatment on cell yield in bovine NP cell isolation with 6 h of digestion; (B) Effect of collagenase concentration and reaction duration on cell yield. (mean ± SD; n = 10; from three batches of bovine tails; biological duplicate in batch 1, biological duplicate and counting duplicate in batches 2 and 3).

(supplementary file). From Figure 3A, the NP cells started to attach and spread on the culture surface at around day 4 and the cells spread more on the culture plate at day 7. This phenomenon was similar to the attachment of trypsinzed cells during cell culture passaging (Fig. 3B) but was slower than trypsinzed cells which may attach to the culture surface again within a day. Besides, heterogeneity in attachment time was observed for the isolated cells.

Requirement of Pronase in Rat NP Cell Isolation

NP cells could be isolated from bovine NP tissue even without the use of pronase (Fig. 2). The difference in cellular arrangement (as shown in Figure S8 of the supplementary file) prompted us to investigate the requirement of pronase in rat NP cell isolation. Unlike from bovine NP, cells could not be released from the rat NP tissue without the use of pronase (Fig. 4). This suggested that pronase is required for rat NP cell isolation, especially when short digestion time is used.

Effect of Different Isolation Protocols on RT-qPCR

In Table 3, all of the chosen protocols yielded cells of quality suitable for RT-qPCR (260/280 >1.8 and 260/230 >1). In these sets of experiments, a higher collagenase concentration (1 or 2%) was used to release the cells in shorter time. Among the chosen conditions, RNA extracted from cells with 1% simultaneous collagenase (1%) and pronase (0.1%) digestion for 3 h had the highest RNA yield of about 5.81 μg RNA per g of NP tissue (Table 3). However, the difference was not statistically significant and different protocols under test could provide RNA of reasonable yield.

There were slight changes in relative gene expression for cells isolated with different protocols but NP cells still had higher chondrocyte and NP-marker expression compared with AF and muscle. Figure 5 showed the relative mRNA levels detected for RNA extracted from cells isolated with different protocols. With longer digestion time (3 h instead of 2 h with 1% collagenase), there might be lower relative mRNA levels of *CDH2* ($p = 0.24$) and *KRT18* ($p = 0.07$). Digestion with higher collagenase concentration (2% instead of 1%) might reduce the mRNA levels of *ACAN* ($p = 0.10$) and *COL2* ($p = 0.29$). There was no significant difference in relative mRNA levels for NP cells isolated with or without the use of pronase. Despite the variations in relative mRNA levels in cells isolated with different isolation methods, the *ACAN* and *COL2A1* mRNA levels were higher in NP and AF cells compared with muscle cells. Besides, NP cells obtained from different protocols had higher *CDH2*, *KRT18*, and *KRT19* expressions than AF cells.

DISCUSSION

More Living Cells Were Observed in NP Tissue Than AF Tissue Before Cell Isolation

In Figure 1, more dead cells were observed in AF tissue than NP tissue. One of the reasons may be the cells were dying after the animal was dead and the cells in AF, which in general had more access to nutrient, died more easily. In contrast, the NP cells which originally were in tissue with low nutrient supply and hence were able to survive for a longer time after the animal was dead. Another reason for this phenomenon was that antiseptic was used to sterilize the tail surface before the tissue harvest and the antiseptic may diffuse into the AF more easily and kill the cells, causing the relatively high dead cell percentage in the AF tissues.

Effect of Pre-Collagenase Treatment

Trypsin was used in cell isolation from rat NP tissue[14] and human articular cartilage.[15,16] Nevertheless, the

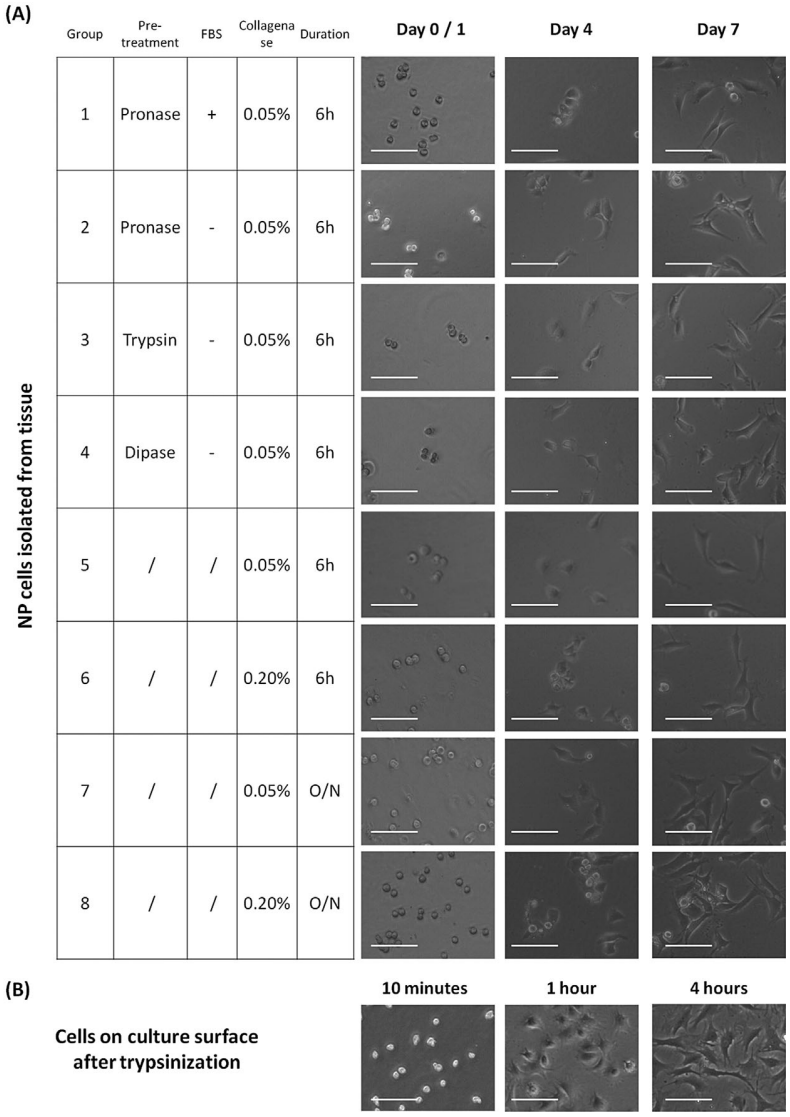


Figure 3. The difference in cell attachment speed between NP cells isolated from the tissue and NP cells during passaging. (A) Micrographs showing NP cells isolated with different conditions and cultured in monolayer at day 0 (day 1 for the samples with overnight digestion), day 4, and day 7 from the day of tissue harvest. (B) Micrographs showing cells cultured for 10 min, 1 and 4 h after trypsinization and re-plating in passaging (scale bar = 100 μm).

use of trypsin or dispase did not increase the cell yield from bovine NP tissue digestion. Thus it is not recommended to use trypsin or dispase in bovine NP cell isolation. On the other hand, the collagenase solution used in tissue digestion may also contain other enzymes. For example, collagenase type 2 from Worthington (cat# LS004177) has clostripain activity. Collagenase from Clostridium histolyticum from Sigma (cat# C5894) contains clostripain, nonspecific neutral protease and tryptic activities and collagenase from Gibco (cat# 17101-015) contains clostripiopeptidase A and a number of other proteases, polysaccha-

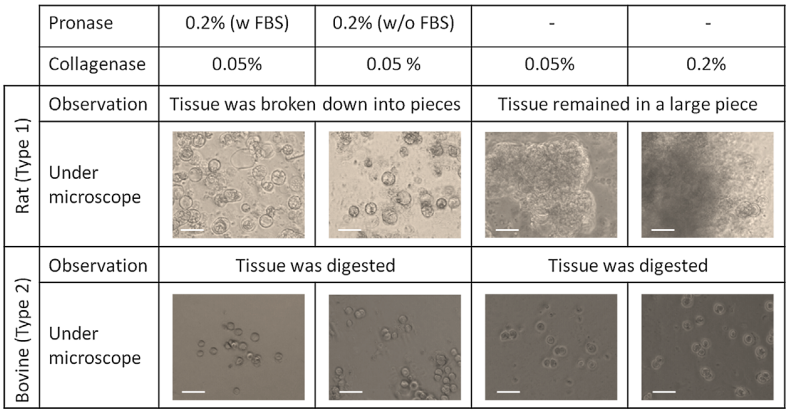


Figure 4. The requirement of pronase in rat NP cell isolation. For bovine NP, 1 h of pronase digestion followed by 5 h of collagenase digestion or 6 h of collagenase digestion. For rat NP, 1 h of pronase digestion followed by 2 h of collagenase digestion or 3 h of collagenase digestion. Shorter digestion time was used for rat NP due to its smaller disc size and the general shorter digestion used in reported studies. (Scale bar = 50 μm). Further information is given in the supplementary file.

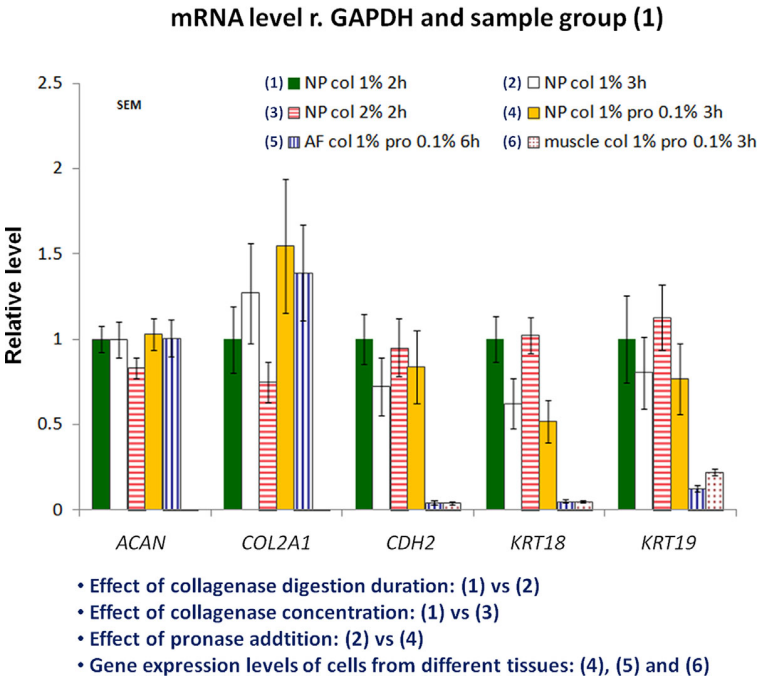


Figure 5. Effect of different isolation protocols on the relative mRNA levels in bovine cells detected by RT-qPCR (mean ± SEM; n = 12; 2 batches of cell isolation; biological triplicates and technical duplicates in each batch except for AF where n = 10).

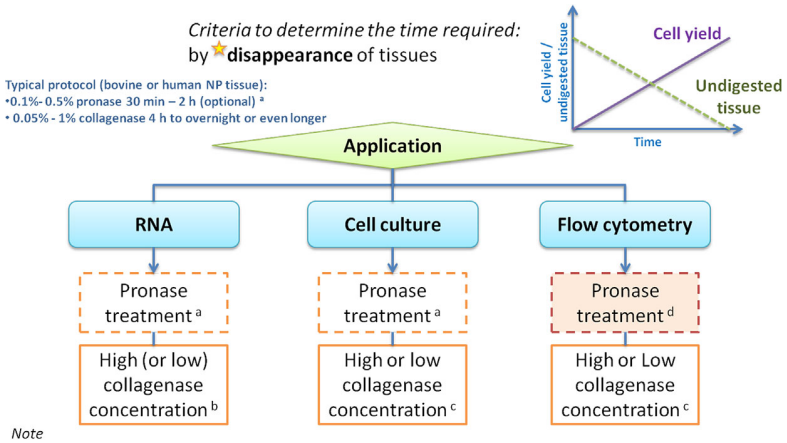
idases, and lipases. The presence of other proteases in the collagenase preparation may also explain why the use of trypsin or dispase did not enhance the cell yield.

Optimal Protocols for Different Applications

The suggestions in deciding the isolation protocols were summarized in Figure 6 and explained in the following.

Disappearance of Tissue as an Indicator of Cell Yield

The components of the IVD changes with ageing and degeneration.[17] NP has a higher percentage of proteoglycans while AF has a higher percentage of collagen[17] but the differences between the outer AF and inner NP become less distinct in adult discs compared to young discs.[18] Therefore the NP or AF tissues obtained from human or other animals at different ages may have different matrix components. In our study, a digestion protocol (0.2% pronase for 2 h and 0.05% collagenase overnight) was sufficient to digest the bovine NP tissue but not the AF tissue (Figure S9 in the supplementary file). This implies the need of deciding the digestion protocol based on the samples. Here, we propose that the disappearance of tissue may be used as an indicator of the cell yield and to determine the tissue digestion duration (as explained in Figure S10 in the supplementary file). As shown in Figure 7, the number of isolated cells increased when



Note

a. Pronase treatment may shorten the required digestion time. It is required for tissue with high cell-cell contact (eg. rat, mouse NP). It is optional for bovine NP tissue without cell clusters. For dissociating cells in clusters, pronase is required and it may be used (again) after the tissue has been digested using collagenase.
b. Depends on the genes under study (the mRNA levels of some genes may change upon prolonged digestion).
c. A higher collagenase concentration requires shorter digestion time but needs more enzyme. A lower collagenase concentration requires longer digestion time but is less costly.
d. Pronase (or collagenase) treatment may damage cell surface markers. Verification may be required to ensure negligible changes due to enzyme treatment for the markers under investigation.

Figure 6. Suggestion about the cell isolation protocols for different applications (RNA extraction for gene expression profiling, cell culture, and surface marker characterization using flow cytometry). In general, the cells are released from the tissues when they are digested. Thus the cell yield increases with the disappearance of undigested tissues and this can serve as a criteria to determine the time required for digestion during the cell isolation. Further information is given in the supplementary file. The filled box of pronase treatment for flow cytometry indicates that the use of pronase in cell isolation may influence the reliability of results in flow cytometry analysis.

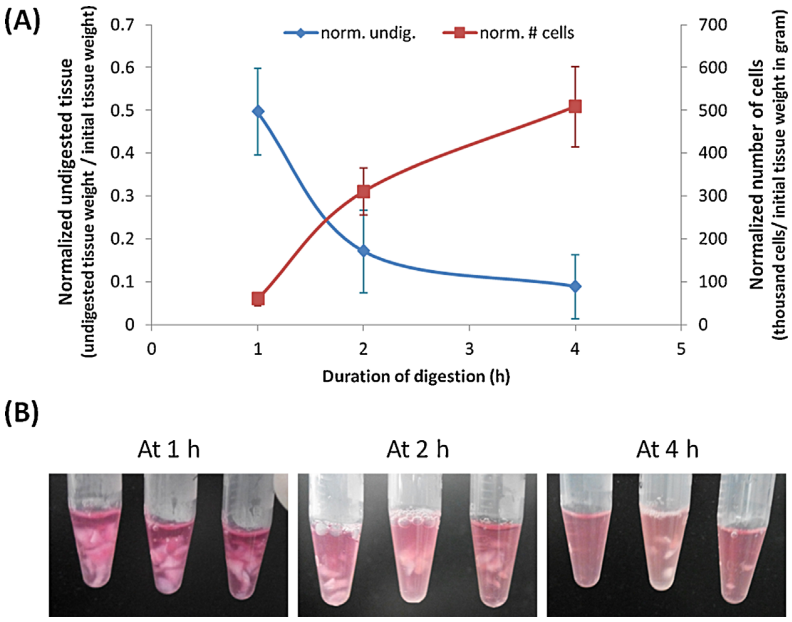


Figure 7. The relationship between the amount of undigested tissue and the number of isolated cells. (A) Plot of normalized amount of undigested tissue and the number of isolated cells vs duration of digestion in 0.2% collagenase. (mean ± SD, n = 3) (B) Photos of digestion captured at 1, 2, and 4h. The NP tissue disappeared with time and the number of isolated cells increased meanwhile.

the amount of undigested tissues decreased. In addition, using bovine AF and tendon tissue as examples, we showed that the cells retained in the undigested tissues can be released by further digestion of the tissue (Figure S11 and Figure S12 in the supplementary file).

Isolation of Cells for RNA Extraction for Phenotyping

For isolation of cells for RNA extraction, it may be preferred to use shorter time for digestion for some studies as RNA may start to degrade when the animal was sacrificed. Sharova et al. has shown that the average half life for mRNA is about 7.1 h in mouse embryonic cells and the half life is longer for genes related to metabolism and structure such as extracellular matrix and cytoskeleton while genes with regulatory functions such as transcription factors may have half-life less than 1 h.[19] Among our chosen markers, *ACAN* and *COL2A1* are genes for extracellular matrix while *KRT18* and *KRT19* are related to intermediate filament which is a type of cytoskeleton. Thus these makers were comparatively less affected by the degradation process during the digestion.

There were only a few studies studying RNA from NP tissues and there is no standardized protocol to date (summarized in Table 4). In this study, we also tested cell isolation with a lower collagenase concentration overnight and the results are given in the supplementary file.

Isolation of Cells for Primary Culture

A comparatively wide range of protocols can be used for isolating cells for cell culturing purpose. High cell viability was obtained even after 0.2% collagenase treatment for overnight for bovine NP tissue digestion (Fig. 2B). In other words, 0.2% collagenase did not cause serious cell damages. However, the cell yields of 0.2% collagenase digestion of 6 h or 0.05% for over-

Table 4. Studies That Investigated the Gene Expression in IVD Tissues Through Cell Isolation with Sequential Digestion Using Pronase/ Protease and Collagenase

Tissue	Pronase/ Protease	Collagenase	Extra Enzyme[a]	Extraction	Reference
Bovine NP and AF	0.5%, 1 h	0.5%, 2–3h	H	TRIzol	Minogue et al.[26]
Human NP	0.2%, 1 h	100 U/ml, 8h	D	Modified TRIspin	Rutges et al.[50]
Human NP	2 U/ml, 30 min	0.2%, 4 h	/	TRI-reagent, MinElute Cleanup	Phillips et al.[51]
Human NP	0.25%, 40 min	0.025%, 4 h	/	TRIzol	Liu et al.[52]
Human NP	0.5%, 1 h	0.5%, 2-3 h	H	TRIzol	Minogue et al.[53]
Human AF and AC	0.2%, 1 h	200 U/ml, O/N	D	Modified TRIspin	Rutges et al.[50]
Rat NP	0.2%, 1 h	0.04%, 8 h	D	Modified TRIspin	Lee et al.[54]
Rat AF	0.2%, 1 h	0.1%, O/N	D	Modified TRIspin	Lee et al.[54]
Rat AC	0.2%, 1 h	0.1%, O/N	/	Modified TRIspin	Lee et al.[54]

[a]H denotes hyaluronidase and D denotes DNase.

Table 5. Cell Density Estimation from Paraffin Sections of Human IVD Tissues (Mean ± SD, Two or Three Patients per Group and Two or Three Sections per Patient)

Sample	Age	Planar Density (per mm^2)	Volumetric Density (per mm^3)
Scoliotic NP	13, 15, 15	69.6 ± 49.9	679 ± 662
Scoliotic AF	13, 15, 16	42.1 ± 16.5	287 ± 165
Degenerated NP	47, 49	33.0 ± 12.9	198 ± 118

night were also higher compared to 0.05% for 6 h. Thus we may use high collagenase concentration with shorter time or low collagenase concentration for longer time to suit our other experimental schedule or cost consideration. As each batch of enzyme preparation may not be the same and there are many other factors which vary from laboratory to laboratory and may influence the actual tissue digestion rate, it is desirable to monitor the disappearance of the tissues rather than simply following a fixed protocol optimized by others. In general, the cell yield is closely related to the disappearance of the tissues.

Isolation of Cells for Characterization by Flow Cytometry

In cell characterization by surface markers, the use of pronase may not be preferred as it may damage the

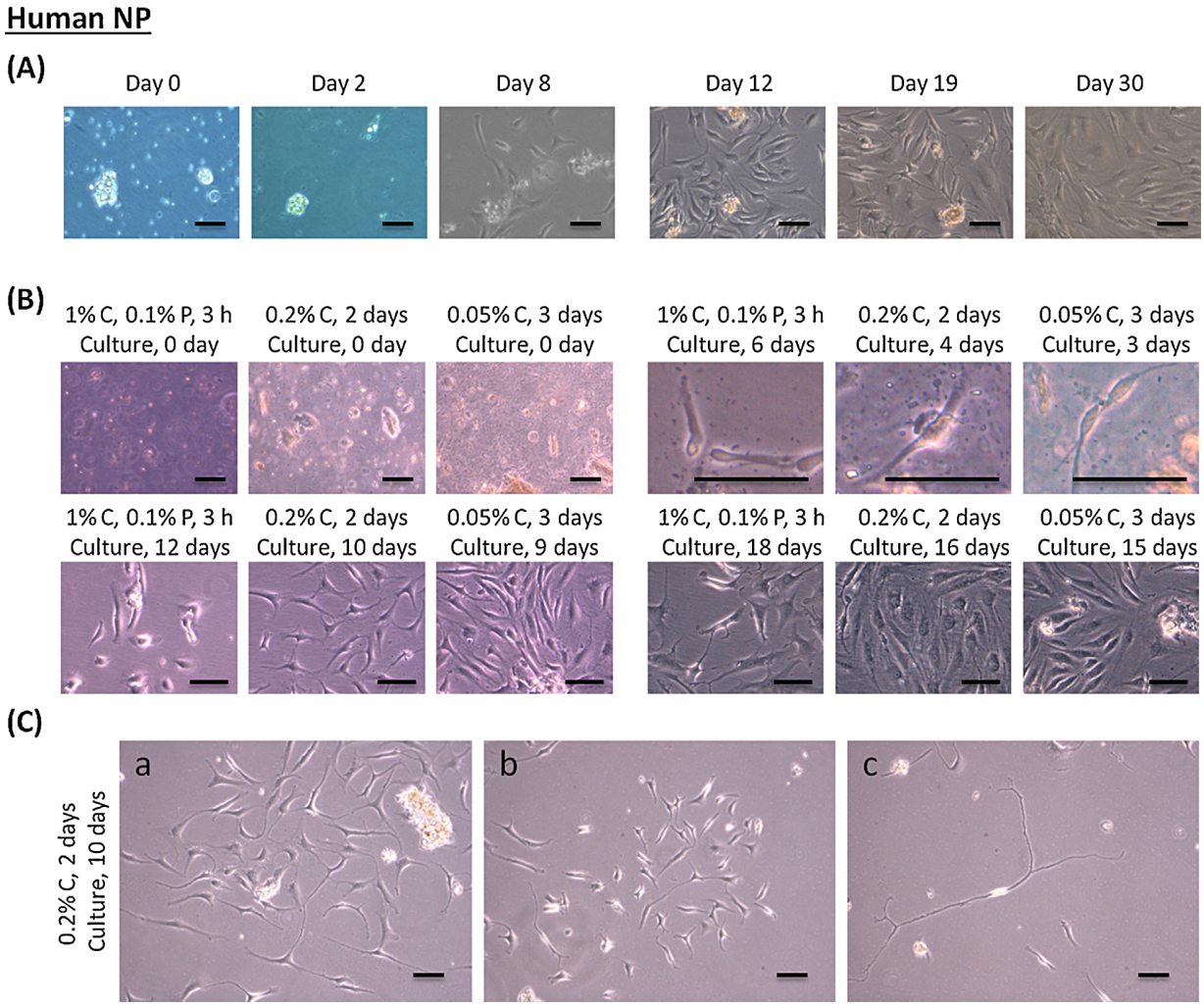


Figure 8. Micrographs of isolated human NP cells. (A) Cells isolated from human gel-like NP tissue from a 42-year-old donor with overnight digestion in 0.3% collagenase and cultured in monolayer at day 0, day 2, day 8, day 12, day 19, and day 30. (B) Cells isolated from the NP of a 68-year-old donor with 1% collagenase and 0.1% pronase for 3 h, 0.2% collagenase for 2 days or 0.05% collagenase for 3 days and cultured for different numbers of days. (C) Cells isolated from NP of a 68-year-old donor with 0.2% collagenase digestion for 2 days and cultured in monolayer for 10 days, showing different morphology of cells. a. For cells isolated with collagenase and without pronase, there may be clusters of cells after tissue digestion and these clusters may attach to the cell culture surface upon culturing. b. At day 10 of monolayer culture, some cells spread on the culture surfaces with different degrees of spreading. Some cells had long processes protruding from their cell bodies. c. A cell with comparatively long processes protruding from its cell body. (scale bar = 100 μm) (more photos of the isolated NP, AF, and CEP cells are given in the supplementary file).

surface proteins due to its relatively low substrate specificity. A number of studies have been performed to investigate the effect of pronase in different applications and reported that proteases affected the cell properties of Lymnaea neurons,[20] extracellular matrix of chondrocytes,[21] lipopolysaccharide receptor of Kupffer cells,[22] and Fc IgG receptors, CD16 and CD32 of lymphocytes[23,24] (details given in the supplementary file). Thus we also tested the effect of pronase on surface antigens using human mesenchymal stem cells (MSC) from bone marrow. Fujita et al. have identified a surface protein CD24 that is specifically expressed in rat NP cells[25] but there is no verified NP surface marker for bovine system to date. Thus human MSC was used to demonstrate the effect of pronase instead of bovine NP cells. The treatment of cells with pronase for 5 min decreased the CD90 antigen by 10% (Figure S13 in the supplementary file). This indicated caution should be made when interpreting the flow cytometry results for cells isolated with the use of pronase since prolonged pronase treatment may yield false negative results. Similarly, some collagenase preparation may also contain some other proteases. Thus cautions are required when analyzing data about cell characterization using surface markers when proteases were involved in the cell preparation. On the other hand, some intracellular markers may be used in cell characterization using flow cytometry after fixation and cell permeabilization.

Isolation of Cells From Human IVD Tissues

As shown in Table 5, the estimated cell density of human NP of 13 to 15-year-old donors is 679 cells per mm^3 which is smaller than that of adult bovine NP (1,530 cells per mm^3, Table 2) and the cell density of degenerated NP from 47- and 49-year-old donors is even lower. The cell density difference between bovine NP and human NP is within one order of magnitude. We tested the isolation of cells from human NP tissue, AF-like tissue and CEP. As shown in Figure 8A, cells could be isolated from the NP of a 42-year-old donor after overnight digestion in 0.3% collagenase and the isolated cells could attach and proliferate when seeded on a culture surface. We also compared the cell isolation using pronase together with collagenase for 3 h, 0.2% collagenase for 2 days and 0.05% collagenase for 3 days with tissues from a 68-year-old male donor. Cells could be obtained with all the three isolation conditions, and could attach to the plate and proliferate (Fig. 8B). Figure 8C shows the isolated NP cells of different morphology attached to a culture surface. Some cells have long processes protruding from the cell bodies. When the human NP tissue was digested with collagenase alone, a mixture of cell clusters, single cells with and without pericellular matrix, and debris was resulted (Fig. 9A). Further digestion with 0.5% collagenase for 40 min did not eliminate the cell clusters but further digestion with 0.2% pronase for 40 min yielded single cells without pericellular matrix (Fig. 9B), implying pronase is effective in breaking cell clusters. Further details of cell isolation from human IVD are given in the supplementary file.

CONCLUSION

In summary, the cell yield from bovine NP tissue can be increased by using high collagenase concentration, long digestion time or with pronase pre-treatment in general. Depending on different purposes, the procedures should be fine tuned with good understanding of the various factors involved in the isolation. For cell culturing, we can obtain a reasonably good yield with a wide range of protocols. In other words, either using a higher collagenase concentration for shorter time or a lower collagenase concentration for longer time may achieve similar yield. The use of pronase prior to collagenase treatment may facilitate the isolation but not absolutely necessary for bovine NP tissue. Protease other than collagenase is required for dissociating

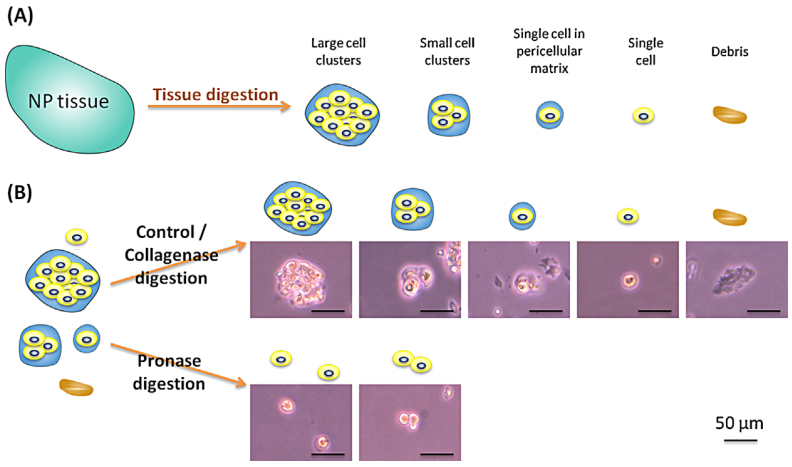


Figure 9. The action of collagenase and pronase. (A) Schematic showing the enzymatic digestion of NP tissue. The digestion of NP tissue may yield large cell clusters, smaller cell clusters, single cells embedded in pericellular matrix, single cells without pericellular matrix, and debris which may be proteins/ non-proteins not digested by the enzymes. When the human degenerated NP tissue from a 68-year-old donor was digested with collagenase alone, there were more cell clusters compared with that digested with the use of pronase. (Photos of cells cultured for 0 day in Fig. 8B) (B) Schematic showing the digestion 0.2% collagenase for 2 days from a 68-year-old donor with further digestion in medium (control), 0.5% collagenase or 0.2% pronase at 37°C for 40 min. When the partially digested tissue was further digested in medium or 0.5% collagenase for 40 min, cell clusters and debris remained in the digestion and cells were still embedded in the pericellular matrix. When the partially digested tissue was further digested with pronase, single cells were obtained and the pericellular matrix around the cells disappeared (Fig. S18 in the supplementary file). Photos of cells after attachment to culture surfaces were also shown in the supplementary file (scale bar = 50 μm).

cells in clusters. For RNA extraction, shorter isolation duration may be preferable depending on the genes being studied. For flow cytometry characterization, cautions are required for cells with pronase treatment for reliable interpretation. Based on this information, researchers can devise their own cell isolation protocols for their applications based on a clear rationale and enhance the efficiency and reliability of the associated experiments.

AUTHORS' CONTRIBUTIONS

In this report, Juliana T. Y. Lee designed and performed the experiments, and wrote the paper; Kenneth M. C. Cheung and Victor Y. L. Leung were involved in experiment design, supported the study and helped in the revision of this paper. All authors have read and approved the final submitted manuscript.

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