

The Use of Collagenase III for the Isolation of Porcine Aortic Valvular Interstitial Cells: Rationale and Optimization

Elizabeth H. Stephens, Joshua L. Carroll, K. Jane Grande-Allen

Department of Bioengineering, Rice University, Houston, Texas, USA

Background and aim of the study: Substantial heart valve research relies on the isolation of valvular interstitial cells (VICs). While a wide variety of conditions have been reported for VIC isolation, the effectiveness of these methods has rarely been compared. It is also likely that valve donor age will influence these valvular tissue dissociation conditions. The study aim was to increase the efficiency and cost-effectiveness of VIC isolation, while taking into account possible differences due to valve donor age. **Methods:** Aortic valves were obtained from six-month-old (n = 24) and six-week-old (suckling) pigs (n = 45) within 24 h of death. After removal of endothelial cells, the tissues were minced and subjected to a variety of enzymatic digestions for variable lengths of time.

Given the severity and prominence of heart valve disease and problems with artificial and bioprosthetic valves, the production of tissue-engineered heart valves to treat heart valve disease is of major importance (1), particularly for the treatment of pediatric valve disease (2). Not only do artificial replacement valves have higher complication rates in children, but pediatric patients must also undergo multiple open-heart surgeries before adulthood to replace at intervals the artificial valves that the child has outgrown (2). While an autologous tissue-engineered heart valve is the ultimate goal because of immune and coagulation issues, recent research has focused on the basic science of valves in order to determine biological design criteria (3,4). This research often relies on valvular interstitial cell (VIC) isolation from the valves of a widely investigated animal model, the pig.

The techniques used to isolate primary cells general-

Results: The optimal concentration of collagenase III was determined as 1 mg/ml for six-week-old pigs, and 2 mg/ml for six-month-old pigs. The optimal duration of digestion was 4 h for both ages. The addition of neutral protease (2 mg/ml) further increased yield, while additional DNase and hyaluronidase had no effect. Yield was not influenced by the volume of enzyme solution, nor the use of previously frozen enzyme solution.

Conclusion: These findings provide age-specific conditions for improving the yield of VIC isolation, which should be of value in experimental studies of valvular cell biology and tissue engineering investigations.

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ly fall into one of three categories: enzymatic; mechanical; and primary explant (5). Primary explant, while avoiding the exposure of tissues to harsh enzymes, selects for a mobile cell phenotype and therefore is only recommended for use when a tissue is extremely limited or fragile (5). Enzymatic and mechanical disaggregation results in a more representative cell population within a shorter period of time (5). In enzymatic disaggregation, a balance is sought between sufficient enzymatic degradation to allow for the release of cells and minimization of the damaging effects of enzymes on the viability of cells. Among the variety of enzymes used, the collagenases and neutral protease (also referred to as dispase) are considered to be 'gentle' enzymes, but provide incomplete disaggregation (5). Trypsin and pronase, on the other hand, are harsher enzymes that produce the most complete dissociation, but are more damaging. The most common enzyme used is trypsin, which refers to a mixture of pancreatic enzymes (also called pancreatin) (6,7). Trypsin is effective and generally well tolerated by a large variety of cell types, and also has the benefit that any residual activity is neutralized by culture media serum (5). However, trypsin is ineffective in fibrous tissues, such

Address for correspondence:
K. Jane Grande-Allen, Rice University, Department of Bioengineering P.O. Box 1892-MS142, Houston, TX 77251-1892, USA
e-mail: grande@rice.edu

as the collagen-rich heart valves, in which collagenase is preferred (5). There are several classes of collagenases: collagenases I and II have high clostripain tryptic activity; collagenase III has lower tryptic activity and thus is less harsh; and collagenase IV is recommended for specific use in the isolation of pancreatic islet cells (7). Additional enzymes are often added to the enzymatic solution, such as DNase, which degrades the DNA from ruptured cells as it can inhibit proteolysis and cause cellular aggregation (5). Hyaluronidase is particularly useful in digesting the glycosaminoglycan (GAG) hyaluronan, which can be prevalent in the extracellular matrix (ECM) surrounding the cells.

For the study of heart valves, reported conditions for cell isolation vary dramatically from time durations of 30 min (4) to 24 h (8), and include a variety of enzymes at different concentrations (Table I). At this point it should be noted that units of enzyme activity vary between manufacturers, although these units are less frequently reported by investigators. Enzymes used in heart valve digestion tend to be various mixtures of collagenases, although no optimization of enzymes and digestion conditions has been published. Furthermore, consideration has not been given to how methods should be modified for cell isolation from valve donors of different ages. Such modification is likely necessary, and in fact has been reported previously by Angrist, who found that valve tissues showed reduced susceptibility to collagenase digestion with advancing age (geriatric) (9). Furthermore, recent studies have shown significant changes in valve composition with age, including the quantity of proteoglycans and GAGs, changes in collagen content and cross-linking (10), and the density of cells (11). Given these differences, it is likely that enzymatic degradation of the ECM of aortic valves from donors of different ages will require different dissociation conditions, whether these donors are human surgical subjects or animal models.

Although optimization studies have rarely been published for valvular cell isolation (12), these experiments have been performed in other tissues. For example, optimization experiments with osteoblasts showed that enzymatic isolation, rather than the primary explant technique, provided the osteogenic differentiation desired (13). In contrast, in the optimization of human gingival cell isolation, better results were found with the direct explant technique rather than enzymatic isolation (14). The effectiveness of different enzymatic mixtures has also been compared in other tissues (15). From these studies it is clear that the optimum dissociation conditions and enzymes used for cell isolation depend on the tissue matrix content and the desired cell phenotype.

It was hypothesized, therefore, that valve tissues from donors of different ages, given their distinct compositions, would require different digestion conditions for optimal yield. It was also hypothesized that there would be a range of conditions that improves not only yield but also cost effectiveness. Therefore, a variety of combinations of enzymatic digestion mixtures (with collagenase III as the base enzyme, for the reasons stated above) and incubation conditions was examined to determine the optimal cocktail for the isolation of cells from porcine aortic valves. Valves from both six-week-old and six-month-old pigs were used to determine the need to tailor dissociation conditions to valve donor age.

Materials and methods

General procedure

Porcine aortic heart valves were obtained from an abattoir (Fisher Ham and Meat, Spring, TX, USA) within 24 h of the animals' death. These valves were dissected and rinsed with phosphate-buffered saline (PBS). For six-month-old valves (n = 24), each aortic

Table I: Reported enzymatic valvular interstitial cell (VIC) isolation techniques.

Enzyme	Concentration	Time	Reference
Collagenase (W)	600 U/ml	Overnight	(20)
Collagenase II (S)	1000 U/ml	30 min	(21)
Collagenase (G)	0.08%=100 U/ml	24 h	(8)
Collagenase II (B)	1000 U/ml	Step 1: 5 min Step 2: 30 min	(4)
Collagenase I (W) and Elastase III (S)	165 U/ml 15 U/ml	Overnight	(22)
Collagenase (B)	0.6 mg/ml	Step 1: 30 min Step 2: 60 min	(23)
Collagenase (S)	0.16%*	2 h	(12)

*The unit concentration could not be determined.
 B: Boehringer; G: Gibco; S: Sigma; W: Worthington.

valve leaflet was further cut in half, with each half acting as a sample (total 137 samples); for the six-week-old (suckling) valves (n = 45), a whole aortic valve leaflet constituted a sample (total 134 samples). In both cases the wet weight of the sample was recorded.

In order to remove the endothelial cells, the tissue was incubated for 30 min in collagenase II (2 mg/ml of a 246 U/mg stock solution; Worthington Scientific, Lakewood, NJ, USA) and 2.5% antibiotic/antimycotic (ABAM; from a stock concentration of 10,000 IU penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B; Mediatech, Herndon, VA, USA) in DMEM (Mediatech) in a shaking incubator (160 rpm, 37°C). The loosened endothelial cells were then removed by gently brushing the leaflet surfaces with a sterile cotton swab; it has been shown previously in the authors' laboratory that this treatment removes endothelial cells (the final cell population does not stain for CD31) (16). The tissue was finely minced and placed into the enzymatic mixture being tested (Table II). The tissue in solution was placed in a shaking incubator (160 rpm, 37°C) for a variable number of hours (Table II). Subsequently, the mixture was filtered through a 70-µm cell strainer (BD Falcon, Bedford, MA, USA). The cells were pelleted by centrifugation (1500 × g, 5 min) and resuspended in 1 ml DMEM:F12 with 10% bovine growth serum (Hyclone, Logan, UT, USA), with 1% ABAM and 5.6 ml/500 ml HEPES buffer (Mediatech).

Assessment of cell number: MTT assay and trypan blue exclusion

To determine cell yield, either an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]; Sigma-Aldrich, St. Louis, MO, USA] assay or a hemocytometer (trypan blue exclusion) was used immediately after cell isolation, as well as 48 h later to determine if the harvested cells had maintained their viability. Trypan blue exclusion was used only in the initial experiment; all subsequent experiments used the MTT assay which offered more precision and efficiency. Manual counting of cells using the trypan blue exclusion method was performed using a hemocytometer according to standard techniques. For each MTT test, 130-µl aliquots of the resuspended cell mixture were seeded in duplicate in sterile 24-well plates; based on initial counts this volume contained approximately 100,000 cells. The MTT assay consisted of adding MTT reagent (5 mg/ml sterile MTT in PBS), gently mixing by rocking the plate back and forth, and then storing in the incubator for 3 h. A 1-ml aliquot of MTT solvent (1 M HCl in isopropyl alcohol) was then added and each well triturated thoroughly. Triplicates of the assay solution from each well were measured in a 96-well plate. Plates were read using a spectrophotometer (SpectraMax M2; Molecular Devices, Sunnyvale, CA, USA) at two different wavelengths

Table II: Experimental conditions.

Duration (h)	Enzyme concentration (mg/ml)			Other factors		
	Coll III	Hyase [*]	DNase	NP	Volume (ml)	Freezing
Six-month-old						
2,4,o/n	1,2,5,10	-	-	-	-	-
4,6	1,2,4,7	-	-	-	-	-
4	2	-	-	-	-	Fresh, Frozen
4	2	0.1,0.2,0.5	0.1	-	-	-
4	2	0.2	0,0.1, 0.2,0.33	-	-	-
4	2	-	-	0.5,2,5	-	-
4	2	-	-	-	-	-
Six-week-old						
2,4,o/n	1,2,5,10	-	-	-	-	-
4,6	1,2,4,7	-	-	-	-	-
3,4,5	1	-	-	-	-	-
4	1	-	-	-	8,20	-
4	1	-	-	-	-	Fresh, Frozen
4	1	0.1,0.2,0.5	0.1	-	-	-
4	1	0.2	0,0.1,0.2,0.5	-	-	-
4	1	0.2	-	0.5,2,5	-	-

^{*}Concentrations are on top of baseline (0.1 mg/ml HA).

Coll: Collagenase; Hyase: Hyaluronidase; NP: Neutral protease; o/n: Overnight.

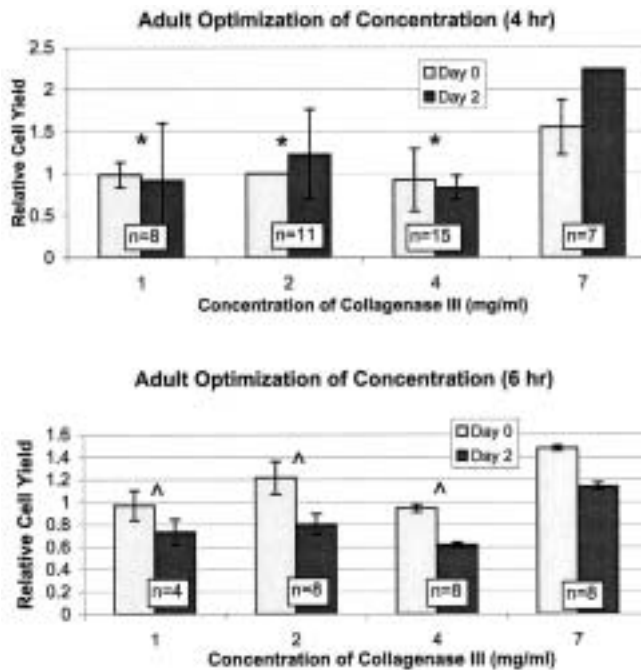


Figure 1: Optimization of collagenase III concentration for six-month-old valves. Upper: 4-h digestion duration, showing MTT cell yield on day 0 and day 2: $p < 0.001$; *, $p < 0.01$ versus 7 mg/ml. Lower: Cell yields resulting from 6-h digestion duration: $p < 0.001$; ^, $p < 0.005$ versus 7 mg/ml. Relative cell yield = yield for each combination of enzyme concentration and digestion duration normalized to the MTT result from the 2 mg/ml and 4-h data.

(A570-A690). The difference in readings at the two wavelengths for each well was taken as directly proportional to the cell number. Both methods were normalized first to tissue weight to obtain cell density. To compare data between different experiments, the cell densities resulting from the different collagenase III concentrations and digestion durations were normalized to the 1 mg/ml (6-week-old) or 2 mg/ml (6-month-old)/4 h results from that same experimental group.

Experimental conditions

The baseline enzymatic cocktail consisted of 2.5% ABAM, 2.5% HEPES buffer, and 10 mg/100 ml hyaluronidase (499 USP/NF U/mg; Worthington Scientific) in DMEM, as well as collagenase III (149 U/mg; Worthington Scientific) at varying concentrations. To test the effect of digestion duration, the minced valve tissues were incubated for 2, 3, 4, or 6 h, as well as overnight (Table II). To test the effect of collagenase III concentration, 1, 2, 4, 5, 7, and 10 mg/ml solutions were evaluated. Under the rationale that an increased volume may allow more opportunity for enzyme-tissue interaction, the effect of the volume of

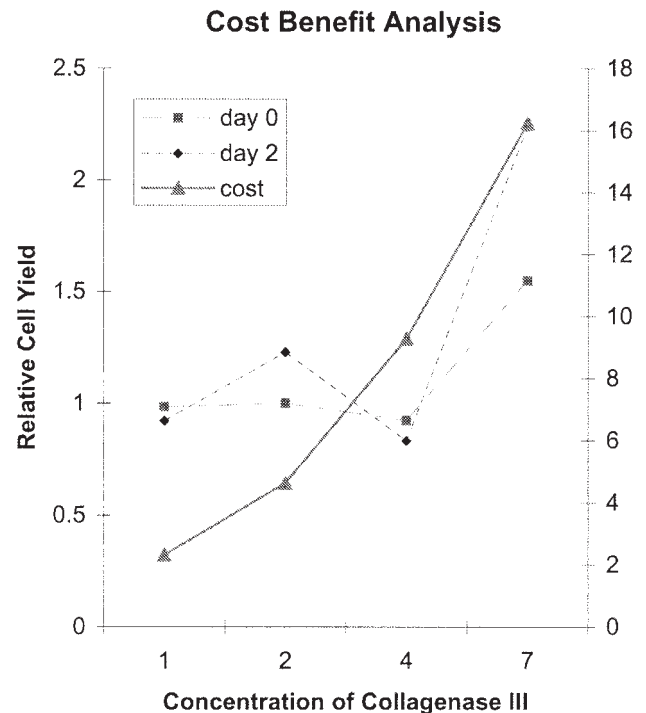


Figure 2: Cost-benefit analysis showing that the cell yield and cost per sample curves cross at approximately 3 mg/ml. As 4 mg/ml gave poorer yields than 2 mg/ml, the lower concentration of collagenases III was selected as optimal.

solution added to the minced tissue was tested by using a digestion mixture of 20 ml (instead of the standard 8 ml) in one experiment. To test the effect of freezing the enzyme mixture before use, one digestion mixture was frozen for several days and then thawed before use in an experiment where it was compared to a freshly prepared mixture of identical composition. Additionally, various concentrations of additional enzymes were tested to determine if they would improve cell yield (Table II). These supplemental enzymes included hyaluronidase (an additional 0.1, 0.2, and 0.5 mg/ml on top of the baseline 0.1 mg/ml), DNase (0.1, 0.2, and 0.5 mg/ml), and neutral protease (NP; 0.5, 2, and 5 mg/ml; 0.28 U/mg; Worthington Scientific).

Statistical and cost-benefit analyses

Multifactorial analyses of variance tests were performed using SigmaStat (SPSS, Chicago, IL, USA) to determine if concentration and duration effects were significant ($p < 0.05$). Post-hoc Tukey tests were performed as needed to compare subgroups. Calculations of cost were based on 2006 Worthington Scientific prices for enzymes and assumed equivalent volumes of enzymatic mixtures per sample.

Table III: Optimized enzymatic digestion mixtures for six-week-old and six-month-old porcine heart valves.

Step 2*	Six-week-old	Six-month-old
Solution component		
Collagenase III	1 mg/ml	2 mg/ml
Hyaluronidase	0.3 mg/ml	0.1 mg/ml
DNase	0.1 mg/ml	-
Neutral protease	2 mg/ml	2 mg/ml

*Step 1 for both age groups loosens the endothelial cells and the mixture contains collagenase II (2 mg/ml of 246 U/mg) and 2.5% antibiotic/antimycotic (from stock concentration of 10,000 IU penicillin, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B) in DMEM.

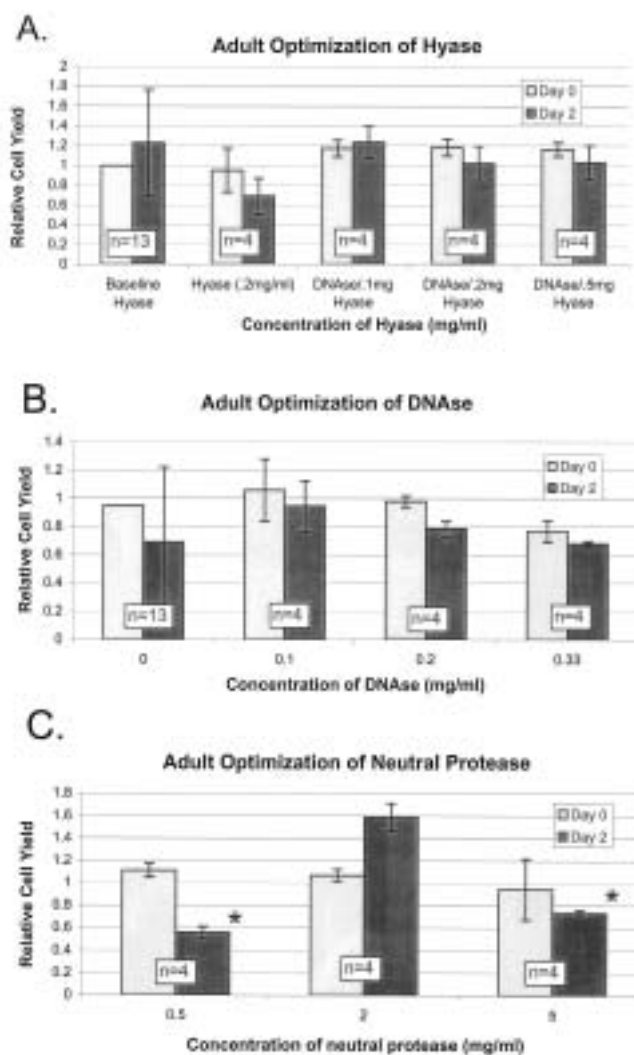


Figure 3: Optimization of additional enzymes for six-month-old valves. A) Optimization of hyaluronidase. B) Optimization of DNase. C) Optimization of neutral protease, for day 2: $p < 0.001$; *, $p < 0.001$ versus 2 mg/ml. For all experiments each sample (n) consisted of one half of an aortic valve leaflet. Hyase: Hyaluronidase.

Results

VIC cell isolation from six-month-old pigs

Initial studies showed that, at low concentrations of collagenase III (1-2 mg/ml), the overnight digest gave the most cells compared to the 2- and 4-h digests, up to 10-fold higher for 1 mg/ml overnight compared to 2 h. The cell yield was greatly increased (average ~4x) with higher concentrations of collagenase III (5-10 mg/ml). However, at the high concentrations the overnight digest yielded lower cell densities (~75%) than 2-or 4-h incubations. Based on this initial study, the overnight digestion was omitted and only shorter digestion

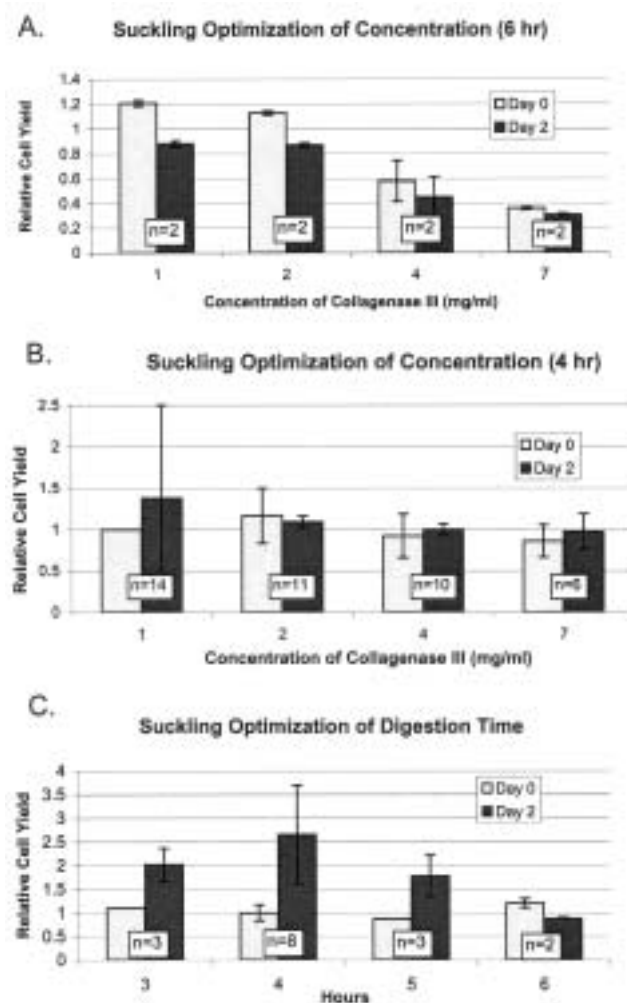


Figure 4: Optimization of digestion duration and collagenase III concentration for six-week-old valves. A) Optimal collagenase III concentration for 6-h digestion duration was 1 mg/ml, $p = 0.038$. B) Optimization of collagenase III concentrations in 4-h digestion duration. C) Optimization of digestion time. For all parts of figure, each sample (n) consisted of one whole aortic valve leaflet. Relative cell yield = yield for each combination of enzyme concentration and digestion duration normalized to the MTT result from the 1 mg/ml and 4-h data.

times were used. This same experiment also showed that most collagenase III concentrations on average yielded a higher (1-2x) cell density at 4 h than after 2 h. For that reason, subsequent experiments compared the effectiveness of 4-h and 6-h digestions. Given that the day 2 analysis of cell viability indicated that the 4-h

digestion culture consistently contained a higher number of cells than the 6-h digestion culture, 4 h was determined to be the optimum digestion duration ($p < 0.001$; Fig. 1). While the greatest yield was obtained with the highest concentration of collagenase III (for 4-h digestion $p < 0.001$; $p < 0.01$ for 7 versus each of 1, 2, and 4 mg/ml), taking into account the cost (Fig. 2), 2 mg/ml was determined to be the optimal collagenase III concentration for the six-month-old valve. Additional hyaluronidase by itself or in combination with DNase did not increase the yield above that of the baseline cocktail (Fig. 3). Similarly, the addition of

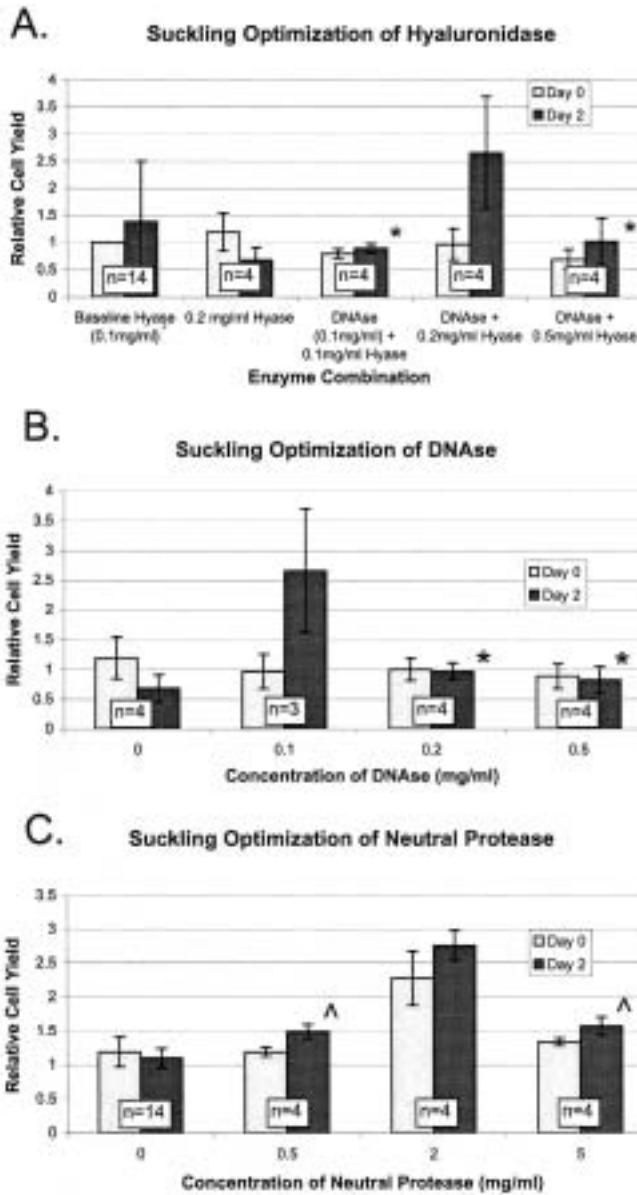


Figure 5: Optimization of additional enzymes for six-week-old valves. A) Optimization of hyaluronidase concentration, for day 2: $p < 0.001$; *, $p < 0.001$ versus DNase + 0.2 mg/ml hyaluronidase. B) Optimization of DNase, for day 2: $p < 0.001$;*, $p < 0.001$ versus 0.1 mg/ml DNase. DNase was in addition to 0.2 mg/ml hyaluronidase. C) Optimization of neutral protease, for day 2: $p < 0.001$; ^, $p < 0.005$ versus 2 mg/ml neutral protease. Neutral protease was in addition to 0.2 mg/ml hyaluronidase. For all parts of figure each sample (n) consisted of one whole aortic valve leaflet.

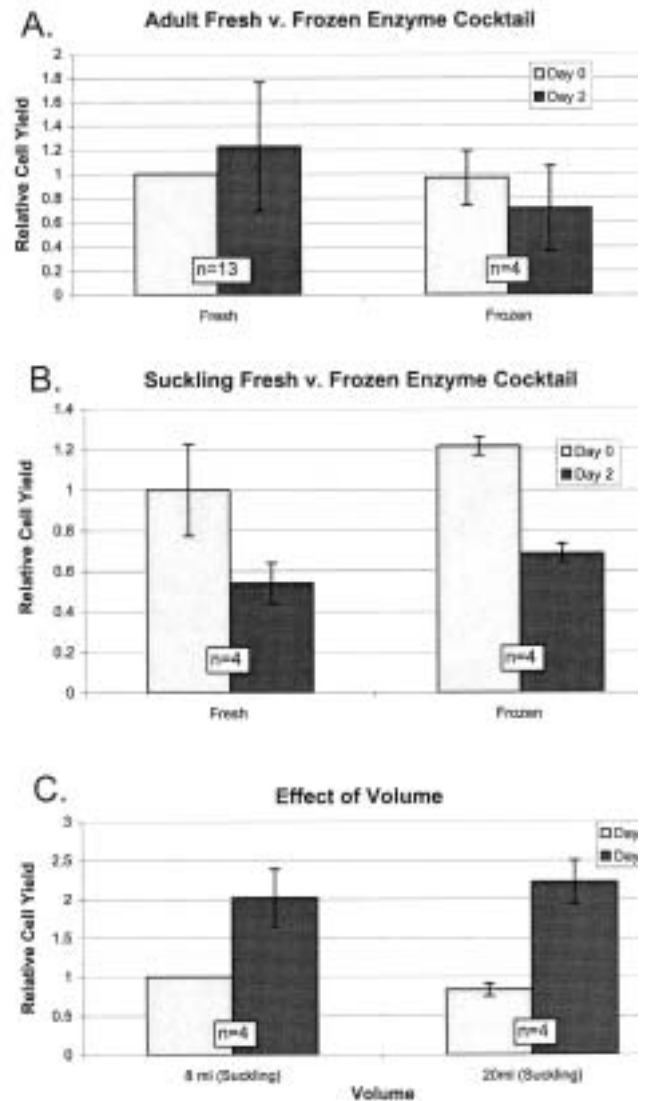


Figure 6: A,B) Fresh versus frozen enzymatic cocktail. C) Effect of volume (performed in six-week-old valves only). Samples in six-month-old valve experiments consisted of one-half of one aortic valve leaflet. Samples in six-week-old valve experiments consisted of one whole aortic valve leaflet.

Table IV: Human and porcine age equivalencies.

Porcine age	Corresponding human age	Reference
5 days	infant	(18)
4 weeks	toddler	(18)
5 weeks	4-5 year-old	(17)
6 weeks	~8 year-old*	
4 months	adolescent	(18)
5 months	13.7 year-old	(19)
6 months	17 year-old female	
	19 year-old male*	
6 year-old sow	old*	
Life span: 10-15 years, but for sows considerably shorter (esp. if reproducing)*		

*Personal communication with Dr. Gil Costas, DVM, Texas Heart Institute.

DNase did not result in a higher yield compared to baseline at any concentration, but NP at a concentration of 2 mg/ml resulted in a significant increase in the number of day 2 viable cells ($p < 0.001$).

VIC isolation from six-week-old pigs

Initial studies in six-week-old valves showed that the yield for the overnight digest was an average of approximately 50% less than the 4-h digest for all concentrations. These studies also showed that, for most concentrations, the yield for the 4-h digest was higher (~1.2-5 \times) than for the 2-h digest. Subsequent experiments, therefore, included 3-, 4-, 5-, and 6-h digests. The optimum duration for digestion for six-week-old valves was found to be 4 h, because this gave the greatest number of viable cells on day 2 (Fig. 4). In six-week-old valves the optimal concentration appeared to be, by a small margin, 1 mg/ml ($p = 0.08$). Given this result, and considering the cost of the enzyme, 1 mg/ml collagenase III was determined to be the optimal concentration. Of the additional enzymes used to supplement the digestion mixture (hyaluronidase, DNase, and NP), the optimal hyaluronidase concentration was 0.2 mg/ml (for a total of 0.3 mg/ml) ($p = 0.024$; $p < 0.001$ for 0.2 versus 0.1 mg/ml). The optimum DNase concentration was 0.1 mg/ml DNase, which substantially increased the yield ($p < 0.001$; $p < 0.005$ for 0.1 versus both 0.2 and 0.5 mg/ml; Fig. 5). Neutral protease increased yield at a concentration of 2 mg/ml, and improved day 2 viability at all concentrations ($p < 0.001$).

In both age groups, freezing the enzymatic preparation ahead of time did not diminish cell yield (Fig. 6). Likewise, an increased incubation volume (tested with six-week-old valves) did not affect yield (Fig. 6). The

Table V: Activities of enzymes in crude collagenase III preparation.

Specific enzyme	Activity*
Collagenase	130-150
Caseinase	148
Clostripain	1.85-1.97
Trypsin	0.24-0.28

*Expressed as u/mg, dry weight.

optimal disaggregation conditions that were determined for each age group are summarized in Table III.

Discussion

A wide range of collagenase III concentrations was found to be effective in the isolation of VICs from porcine aortic valves, though with different results in terms of cell yield. This finding confirms the notion that, in enzymatic disaggregation, a balance is sought between sufficient enzymatic degradation of the tissue to allow for the release of cells and minimization of the damaging effects of the enzymes on cell viability.

Collagenase III was found to be very effective as the main enzyme in valvular disaggregation; this was as expected, given that collagenase is reported to be particularly appropriate for either delicate tissues or fibrous tissues (5). The collagenases are a family of enzymes composed of differing proportions of clostridopeptidase A (which specifically cleaves Pro-X-Gly-Pro) with other proteases, polysaccharidases, and lipases (7). In general, crude enzymatic preparations (as used in this study) are often more successful in cell isolation, as they are less pure and contain contaminating non-specific proteases that aid in digestion (5). However, higher-purity preparations of enzymes are advantageous for being less toxic to cells and more specific to distinct types of ECM (5). At different points in the enzymatic digestion process, different preparations of collagenase are more appropriate. Within the collagenase family, collagenase II is harsher and has more tryptic clostripain activity than collagenase III (7). Therefore, collagenase II can be used briefly to loosen the endothelial cells, but it is most likely too harsh for a sustained duration of digestion.

Although DNase is known to improve yield by degrading DNA from cells that have ruptured, the DNA both inhibits proteolysis and causes cellular aggregation (5), and consequently this enzyme increased only day 2 viability, and not yield. While the addition of hyaluronidase did not improve six-month-old valve dissociation, it did improve six-week-old valve dissociation, consistent with the increased GAG

content in human infant valves compared to human adult valves (11). Neutral protease, which is a gentler, less-tryptic enzyme (similar to the collagenases (7)), was found to increase yield and day 2 viability for both age groups. Based on developmental milestones and growth studies, a six-week-old pig is equivalent to an approximately eight-year-old human, whereas a six-month-old pig is approximately equivalent to a 17- to 19-year-old human (17-19) (Table IV).

Valves from the different age groups were shown to require different concentrations of collagenases. The need for a higher concentration of collagenase III in the older group was not surprising, considering that many research groups have used the digestion time of valve tissue as an indication of cross-linking (with an increase in digestion time indicating more cross-linking), and reported differences between age groups (9). The cell yield from six-month-old valves also increased with the concentration of collagenase, while six-week-old valve disaggregation was improved with lower concentrations and was optimal at 1 mg/ml, implying that higher concentrations may be too harsh for younger cells. Similarly, the finding that 0.1 mg/ml DNase improved the six-week-old valve disaggregation suggests that these cells are more prone to rupture, and therefore the DNase was able to prevent the released DNA from forming sticky cell aggregates.

Cost-effectiveness was a major factor in determining the optimal enzymatic mixture. In 2006, 100 mg of collagenase III cost \$29 from Worthington Biochemical. Per sample, 1 mg/ml of collagenase III cost \$2.32, while 4 mg/ml cost \$4.64, and 7 mg/ml cost \$16.24. The specialized enzymes were also costly; hence, optimizing their effectiveness should offer substantial savings.

Study limitations

The main limitations in the present study were shown by the day 2 tests, which often indicated a small percentage of cells to have died after seeding; this experimental design also could not indicate long-term viability. However, as many valvular cell studies need to expand these cells in culture, the MTT test on day 2 indicated which technique produces the greatest number of viable, proliferating cells in the short term. Another limitation is that only collagenase products from Worthington Biochemical were used, and results may not translate to enzymes from all other manufacturers. In order to enable comparisons between manufacturers, the caseinase, clostripain, collagenase, and tryptic assay numbers for a given product should be examined. The unit activities of the collagenases used are listed in Table V; these data indicate the tight range of activity present between the two different lots of collagenase III used in this study.

In conclusion, the optimum enzymatic digest mixtures were determined as 1 mg/ml collagenase III for six-week-old pigs, along with 0.3 mg/ml hyaluronidase, 0.1 mg/ml DNase, and 2 mg/ml NP dissolved in DMEM containing 2.5% antibiotic/antimycotic solution and 2.5% HEPES buffer. The optimum mixture for six-month-old pigs was 2 mg/ml collagenase III and 2 mg/ml neutral protease, dissolved in the same DMEM/ABAM/HEPES buffer. The optimized duration of digestion was 4 h for each age of aortic valve. These findings provide age-specific and cost-effective conditions for improving the yield of VIC isolation, which should be of value in experimental studies of valvular cell biology and tissue engineering investigations.

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