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In-bag enzymatic splenic digestion: a novel alternative to manual morcellation?

Eric D. Villarreal, MD,[a,b] *William Preston Hewgley, MD,*[a,b] *Walter H. Lang, PhD,*[a] *Christopher L. Morton, BS, MBA,*[a] *Shenghua Mao, MS, PhD,*[c] *Jianrong Wu, PhD,*[c] *and John A. Sandoval, MD*[d,*]

[a] *Department of Surgery, St. Jude Children's Research Hospital, Memphis, Tennessee*
[b] *University of Tennessee Health Science Center College of Medicine, Memphis, Tennessee*
[c] *Department of Biostatistics, St. Jude Children's Research Hospital, Memphis, Tennessee*
[d] *Division of Pediatric Surgery, Baptist Children's Hospital, Memphis, Tennessee*

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ABSTRACT

Background: Contained in-bag spleen morcellation is a conventional extraction technique for safe spleen removal during laparoscopic splenectomy. Existing data for the use of in-bag enzymatic splenic digestion as an alternative to morcellation are lacking. This proof-of-concept study sought to evaluate the effectiveness of single and combinatorial enzyme digestion of murine spleens.

Materials and methods: Murine spleens were digested with collagenase alone or with combinations of commercially available enzymes (collagenase, elastase, hyaluronidase, neutral protease) to determine their degradation effect. The primary end point was the percentage of mass reduction at 15 and 30 min.

Results: For collagenase alone ($n = 15$), the mean reduction in mass was 14 ± 10% (range: 2%-31%) at 15 min and 30 ± 25% (range: 7%-100%) at 30 min. Using combinatorial dissolution with collagenase, hyaluronidase, and elastase ($n = 8$), the mean reduction in mass was 27 ± 16% (range: 6%-42%) at 15 min and 48 ± 27% (range: 3%-100%) at 30 min. Injecting the enzyme solution into whole spleens ($n = 9$) yielded a mean reduction in mass of 22 ± 13% (range: 9%-42%) at 15 min and 55 ± 31% (range: 9%-100%) at 30 min; mean reduction was 9 ± 13% (range: 0%-39%) at 15 min and 23 ± 13% (range: 3%-53%) with no injection ($n = 12$).

Conclusions: We provide the first demonstration of successful enzymatic murine spleen digestion as an alternative method for in-bag spleen removal during laparoscopic splenectomy. However, the significant cost and quantities of commercial enzyme required for clinical application dampens the enthusiasm for this novel approach.

* *Corresponding author*. Baptist Memorial Medical Group Pediatric Surgery, 6215 Humphreys Blvd, Suite 300, Memphis, TN 38120. Tel.: +1 901 227 9870; fax: +1 901 227 9879.
E-mail address: john.sandoval@bmg.md (J.A. Sandoval).

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Introduction

Splenectomy is frequently performed via minimally invasive surgery (MIS) for various, primarily hematologic, indications in children and adolescents. One limitation of using MIS techniques during splenectomy is the difficulty of safely extracting specimens through small incisions. The most conventional method for spleen withdrawal is a contained extracorporeal in-bag morcellation approach whereby the specimen is placed within an Endo Catch bag and removed from the peritoneal cavity after manual morcellation. Massive splenomegaly (spleen weight ≥500 g) precludes this approach due to the maximum capacity of the Endo Catch bag.[1] Other described bag-related complications include perforation and deployment failure, with a perforation rate of up to 4%.[1] Bag perforation has been reported to lead to the spillage of splenic fragments within the abdominal cavity[2] and even to splenosis and immune thrombocytopenic purpura recurrence after therapeutic splenectomy.[3] Alternative splenic extraction techniques include specimen fragmentation in the abdominal cavity (risk of splenosis), extended trocar incision, mini-laparotomy, Pfannenstiel incision[4,5] or natural orifice specimen extraction in adult females.[6]

Although these methods allow for specimen removal with a minimally invasive approach, some authors have argued that the advantages of laparoscopy are not clear in patients with massive splenomegaly, thereby generating concerns about the safety of this minimal access surgery in children with massive splenomegaly.[7,8] Nevertheless, recent work from Hassan and Ali supports the feasibility and safety of laparoscopic splenectomy for pediatric hematologic disorders, and these authors, among others, consider the MIS approach to be the gold standard regardless of spleen size.[9-12] Given the technical challenges associated with retrieval of large specimens during laparoscopy, our query was whether a targeted in-bag spleen enzyme digestion method would present an alternative option to facilitate specimen removal.

The spleen is known for its complex immune cell repertoire and can also be characterized by its unique extracellular matrix (ECM). Studies performed on the ECM of the spleen show two structurally and functionally distinct types: the interstitial matrix and the basement membrane. The molecular composition of both the interstitial matrix (collagen types I, II, and III, fibronectin, tenascin-C) and basement membrane (laminins, collagen type IV, proteoglycans, nidogen) has been well described and reviewed elsewhere.[13] Because the main splenic structures (capsule, trabeculae, vascular walls, and reticular fibers) contain ECM components, we theorized that digesting collagen and other components within the spleen ECM with collagenase and other ECM-directed enzymes may have value as an extracorporeal in-bag "biologic morcellator" to aid with specimen retrieval. This concept has not been studied with the spleen, but translational work using *Clostridium histolyticum* collagenase (CHC) has evaluated highly purified CHC as a nonhormonal local treatment for diseases characterized by a high content of collagen that can be effectively digested and result in reduced tissue stiffness. Described clinical uses for CHC include uterine leiomyomas (fibroids)[14,15] and multiple fibrotic diseases, including Dupuytren's contracture,[16,17] keloid scars,[18,19] and Peyronie's disease.[20-22] Currently, CHC is approved by the Food and Drug Administration only for Dupuytren's contracture and Peyronie's disease.

The objective of this study was to investigate whether commercially available enzymes can effectively digest the ECM in murine spleen specimens and thereby evaluate the feasibility of developing splenic enzymatic digestion into an alternative specimen extraction technique for laparoscopic splenectomy. This proof-of-principle study would provide a basis for further clinical evaluation.

Materials and methods

Animals

C57BL/6 mice were maintained under barrier conditions, and their spleens were harvested and used for enzymatic digestion. Studies were carried out at St. Jude Children's Research Hospital (SJCRH), and the protocol was reviewed and approved by the SJCRH Institutional Animal Care and Use Committee. A total of 30 spleens were treated and analyzed in the experiments.

Materials

The following enzymes were purchased from Worthington Biochemical Corp. (Lakewood, NJ): type V collagenase (CLS-5; C), hyaluronidase elastase, and neutral protease/dispase. For reference, the 1-g bottle of CLS-5 had assayed enzyme activities of 550 U/mg dw collagenase, 570 U/mg dw caseinase, 2.32 U/mg dw clostripain, and 0.10 U/mg dw tryptic acid. Hyaluronidase activity was 770 U/mg dw, and elastase activity was assayed at 4.41 U/mg dw. Candidate enzymes were chosen based on previous methods used to degrade connective tissues and allow for tissue dissolution and subsequent cell isolation.[23-32] Hank's balanced salt solution plus Ca^{2+} and Mg^{2+} (Gibco; Grand Island, NY) was used as the basic digestion medium. The solution-based conditions are similar to those used in previous protocols directed toward spleen dissolution for the purpose of cell isolation.[23-32] Each spleen or spleen section was incubated in buffer solution before digestion to ensure osmotic equilibration before mass and volume evaluation.

Single and combination solution-based enzymatic digestion of murine spleens

Murine spleens were sectioned in half and weighed to 0.0001-g precision on a laboratory scale. The average dry mass of the halves was 0.0459 ± 0.00873 g (range: 0.0327-0.0684 g). Weighed mass had good consistency among the halves. The specimens were then equilibrated in digestion buffer and subjected to single or combination digestion with CLS-5 (C), hyaluronidase (H), elastase (E), and neutral protease (NP) used in different combinations and concentrations. Appendix Tables A1-A2 show the enzyme combinations, concentrations, and masses used in the study. The combinations were

collagenase + elastase (CE), collagenase + hyaluronidase (CH), collagenase + neutral protease (CNP), collagenase + hyaluronidase + elastase (CHE), collagenase + hyaluronidase + neutral protease (CHNP), and collagenase + hyaluronidase + neutral protease + elastase (CHNPE). The specimens were immersed in digestion culture media (Hank's balanced salt solution) and incubated in a cell culture incubator at 37°C for 15 min before measurement of baseline mass. The specimens were then weighed every 15 min for a total of 60 min during the digestions. Analysis of digestions 1-21 (see Table A.3) identified the enzyme(s) that produced the largest statistically significant reduction in mass within a 30-min interval.

Whole murine spleen digestion with combination and intraparenchymal enzyme injection

After identification of the best-performing enzyme solutions, digestions were performed on whole murine spleens to determine the mass reduction at each time point. Whole spleen specimens were weighed on the same laboratory scale, and the average dry mass was 0.0883 ± 0.0128 g (range: 0.0730-0.1152 g). Similar to the halves, the whole spleens had adequate consistency between the specimens. CHE, CH, and C were used for these digestions plus additional digestions with CE and CHNPE (see Table A.3, digestions 22-40). To determine the effect of enzyme injection relative to immersion alone, eight of the whole spleen digestions involved injecting solution directly into the spleen. In seven of the digestions, 50% of the solution volume was injected directly into the spleen, which was then immersed in the other 50% of the solution; 100% of the solution was injected into the spleen in the last digestion. We hypothesized that direct enzyme injection into the splenic parenchyma would yield a more efficient digestion by degrading the trabecules, vascular walls, and reticular fibers within the spleen itself while the capsule would be degraded by the surrounding extrasplenic solution. These digestions were compared with the 11 remaining whole spleen digestions without enzyme injection for statistical analysis of our hypothesis.

The primary outcome measures of this experiment were the percent change in mass at 15 and 30 min. The mass and volume of each spleen sample were recorded before enzymatic digestion. The volume of all spleen sections was within the reading error of available graduated cylinders and microtubes, so their values were not recorded for analysis. The mass of each sample was measured after pipetting the enzyme solution out of the graduated cylinder every 15 min. The same solution was then added back to the graduated cylinder after measurements. Digestion time was paused during mass measurement to ensure that variation in the time taken for these measurements did not affect the exposure time to the enzyme solution.

Statistical analysis

The Wilcoxon signed-rank test was used to determine the significance of the total percent decrease in mass from baseline. The Wilcoxon rank-sum test for paired data was used to determine the significance of the change in mass at each time interval. Longitudinal analysis was used to determine the change in relative mass over time.

Results

Identification of optimal murine spleen enzyme digestion solution—single versus combination degradation

To test the hypothesis that enzymatic spleen degradation can serve as a feasible alternative to morcellation, we first determined its achievability by demonstrating the proficiency of spleen degradation with C, H, E, and NP in different combinations and concentrations (see Table A.1 for details) at the selected time intervals of 15, 30, 45, and 60 min. Without considering the enzyme solution used for digestion, the spleen sample mass was significantly less than baseline mass at every point in time (15, 30, 45, and 60 min) based on the Wilcoxon signed-rank test ($P < 0.001$). Furthermore, there was a significant mass reduction during each 15-min time interval

Table 1 – Summary of enzymatic exposure on whole murine spleens.

Solution	*n*	Digestion time (min)	Mean % reduction	Standard deviation	Maximum % reduction	Minimum % reduction
C	7	15	8	9	19	+*2
		30	32	33	100	7
CE	4	15	11	4	17	7
		30	26	5	33	20
CH	2	15	10	7	15	5
		30	19	3	21	17
CHE	6	15	29	18	42	+6
		30	49	31	100	3
CHNPE	2	15	9	9	15	2
		30	38	20	52	24

* Indicates mass increase.

(Wilcoxon rank-sum test, $P < 0.05$ for all intervals; ANOVA, $P < 0.001$). The percent change in mass at 30 min significantly correlated with overall digestion on longitudinal analysis ($P < 0.0001$). Because there was insufficient power due to inadequate sample size to perform regression analysis on each solution composition, the percent change in mass at 30 min served as a suitable proxy with which to select the best-performing solutions. ANOVA analysis revealed that the number of enzymes used produced a statistically significant difference in the percent change in mass at 15 and 30 min ($P = 0.0103$ and 0.0195, respectively). C, CH, and CHE caused the largest percentage changes in mass at 30 min for 1, 2, and 3 enzyme combinations (maximum reductions of 53%, 46%, and 60%, respectively, *versus* 26% and 24% for the 2-enzyme combinations of CE and CNP, respectively, and 40% for the 3-enzyme combination of CHNP) and were, thus, selected as the best candidates for the subsequent whole spleen and intraparenchymal digestions. We also performed four extra digestions with CE and two with CHNPE.

The effect of enzymatic exposure on whole murine spleens

Our next objective was to determine the effect of enzymatic degradation on whole murine spleens. We hypothesized that combinations of different enzymes (CH, CE, CHE, CHNPE) would lead to more effective whole spleen degradation than would collagenase alone. Summary statistics for these digestions are found in Table 1. Complete digestion at 30 min was achieved twice, once with collagenase alone and once with CHE.

Longitudinal pairwise comparison shows that a statistically significant difference exists between C and CHE ($P = 0.0207$) and between CE and CHE ($P = 0.0118$); sample sizes for the other combinations (CH plus CHNP and CNP from half-spleen digestions) were too small for pairwise comparison. The overall digestion trends for C, CE, CH, CHE, and CHNPE are illustrated in Figure 1.

The effect of intraparenchymal enzyme injection on whole murine spleens

We next tested the concept of an enzyme injection model that would better mimic the intraoperative scenario of using both intraparenchymal and extraparenchymal splenic capsule dissolution. We hypothesized that an accelerated and more-complete splenic dissolution would be observed with this bimodal digestion method.

For whole spleen digestions using intraparenchymal injection ($n = 8$), the mean reduction in mass was 22 ± 13% (range: 9%-42%) at 15 min and 55 ± 31% (range: 9%-100%) at 30 min, whereas it was 9 ± 13% (range: +6%-39%) at 15 min and 23 ± 13% (range: 3%-53%) with no injection ($n = 11$) (see Table 2). Based on longitudinal analysis, Figure 2 shows injection of the enzyme solution was a significant factor for digestion ($P = 0.0012$). This was confirmed with ANOVA, as P values for enzyme injection were 0.0450 and 0.0063 at 15 and 30 min, respectively.

All digestions

The overall digestion trend was significantly correlated with the number of enzymes used (longitudinal analysis, $P < 0.001$). Longitudinal pairwise comparison showed a statistically significant difference between 0 *versus* any number of enzymes ($P < 0.02$ for all), 1 *versus* 3 enzymes ($P = 0.0339$), and 2 *versus* 3 enzymes ($P = 0.0117$), but not between 1 *versus* 2 enzymes ($P = 0.5411$). ANOVA P values grouped by number of enzymes used were 0.0047 and 0.0504 at 15 and 30 min, respectively. The effect of the number of enzymes used is illustrated in Figure 3.

Descriptive enzyme degradation data for the different enzyme combinations used in all digestions are listed in Table 3. For C alone ($n = 15$), the mean reduction in mass was 14 ± 10% (range: +2%-31%) at 15 min and 30 ± 25% (range: 7%-100%) at 30 min. For CE ($n = 6$), the mean reduction in mass was 8 ± 7% (range: +1.5%-17%) at 15 min and 25 ± 6% (range: 17%-33%) at 30 min. For CH ($n = 4$), the mean reduction in mass

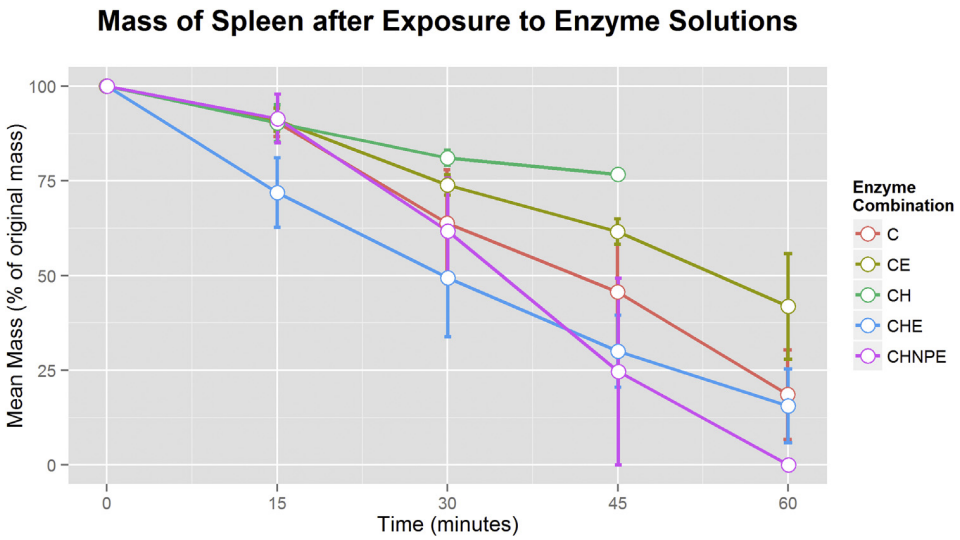

Fig. 1 – Digestion of murine spleen over time with different solutions. (Color version of figure is available online.)

Table 2 – Effect of intraparenchymal enzyme injection on digestion.

Injection	*n*	Digestion time (min)	Mean % reduction	Standard deviation	Maximum % reduction	Minimum % reduction
Yes	8	15	22	13	42	9
		30	55	31	100	9
No	11	15	9	13	39	+*6
		30	23	13	53	3

*Indicates mass increase.

was 12 ± 5% (range: 5%-17%) at 15 min and 28 ± 13% (range: 17%-46%) at 30 min. For CNP ($n = 2$), the mean reduction in mass was 18 ± 3% (range: 15%-21%) at 15 min and 20 ± 5% (range: 17%-24%) at 30 min. For the combination of CHE ($n = 8$), the mean reduction in mass was 27 ± 16% (range: +6%-42%) at 15 min and 48% ± 27% (range: 3%-100%) at 30 min. For the combination of CHNP ($n = 2$), the mean reduction in mass was 15 ± 3% (range: 12%-17%) at 15 min and 35 + 8% (range: 29%-40%) at 30 min. For the combination of CHNPE ($n = 2$), the mean reduction in mass was 9 ± 9% (range: 2%-15%) at 15 min and 38 ± 20% (range: 24%-52%).

Discussion

As a basis for the proposed work, consideration was given to the following clinical parameters for determining practicality of enzymatic splenic dissolution: average intraoperative morcellation time, commercial enzyme availability, and economic feasibility. Safety concerns related to *in situ* enzyme contamination and spillage were not addressed in this project as our main objective was to determine whether enzymatic disaggregation of murine spleen was possible. The significance of our findings in relation to time, materials, and cost are explained in the following, along with considerations for future development of this technique.

Specimen entrapment and the morcellation technique vary between institutions. At SJCRH, morcellation is performed by placing the specimen into a 15-mm Endo Catch Bag that is delivered through a 15-mm umbilical port. This is followed by draping of the surgical field around the port site. The spleen is then manually morcellated by using gynecological ring forceps under direct observation above the abdominal wall. Once the bag containing the specimen has been extracted, laparoscopic access is reestablished, the surgical field is inspected for hemostasis, and any MIS incisions are closed. Our group has not conducted a formal analysis, but we estimate morcellation time to be 10-25 min based on operative cases from laparoscopic total splenectomies performed at our institution (unpublished data). However, the best enzymatic digestion of murine spleens that we found required treatment with the combination of collagenase, hyaluronidase, and elastase for 30 min. Although 30 min was a reasonable target interval given that our intraoperative morcellation times can be up to 30 min, whether it is reasonable to wait for this digestion time could be perceived as inefficient and passive compared with an active manual morcellation process. Consequently, a time-limited digestion protocol would be required for this procedure to be a practical alternative strategy in patients.

Although we demonstrate murine spleen degradation by enzymatic digestion, the ability to upscale the procedure for larger sized specimens may preclude clinical applicability

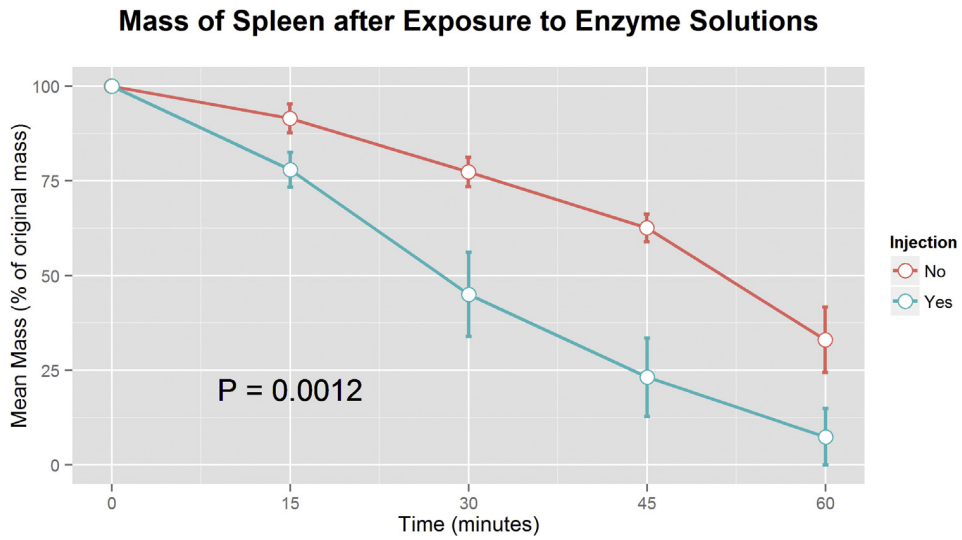

Fig. 2 – Effect of enzyme injection on digestion. (Color version of figure is available online.)

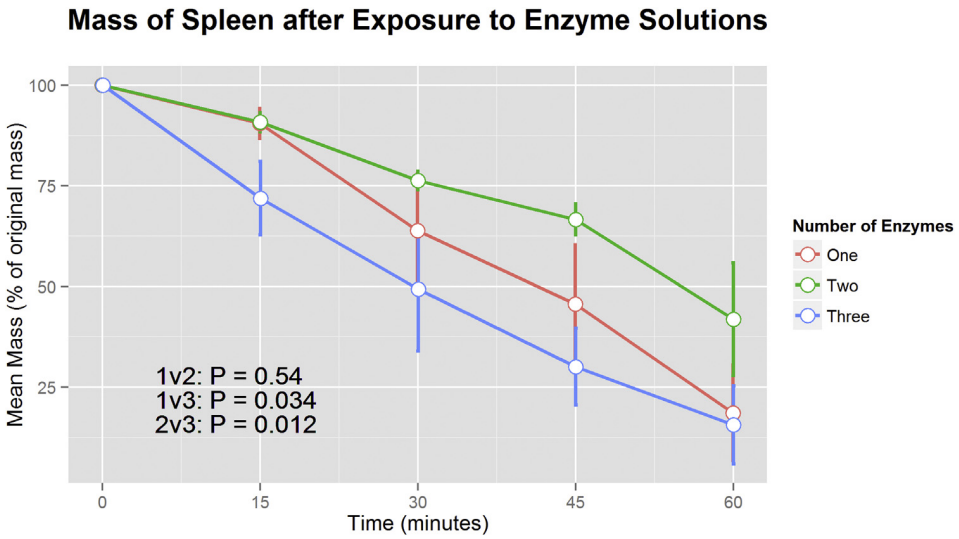

Fig. 3 – Effect of number of enzymes used on mean percent reduction in mass. (Color version of figure is available online.)

when considering the amount and cost of enzyme used per gram of tissue. For murine spleen, published collagenase usage ranges from 100 U/mL per gram of tissue to 300 U/mL per gram of tissue.[33] Although no reported range of enzyme usage is reported for human spleen isolation, massive splenomegaly is defined as ≥500 g (identified by ultrasonography as splenic size ≥500 g by using the formula described by Downey[34]: 0.43 × length × width × thickness = splenic size [in grams]). If a 5-g bottle of collagenase costs $890 ($178/g),[35] the estimated enzyme amount and cost associated with digestion of a 500-g spleen at a spleen to collagenase mass ratio of 3.1 (as used in digestion #32, resulting in complete digestion at 30 min) would be 161 g of collagenase at a cost of $28,480. Thus, costs to attempt enzymatic spleen digestion in a human cohort are exorbitant and cost/supply prohibitive. Nonetheless, before clinical translation can be achieved, the approach presented in this proof-of-concept study will require cost optimization to allow for practical clinical application.

Our analysis used murine spleens as the basis for the feasibility study. An area of further improvement can be envisaged when considering the species differences between the spleen ECMs in humans and nonhuman animal species. The expression differences of collagen type I (Col I), type II (Col II), type III (Col III), type IV (Col IV), vitronectin, fibronectin (FN), laminins, perlecan, heparin sulfates, and hyaluronan in the spleen ECMs from human and nonhuman animal species

Table 3 – Enzyme solution performance for all digestions.

Solution	Digestion time (min)	Mean % reduction	Standard deviation	Maximum % reduction	Minimum % reduction
C	15	14	10	31	+* 2
	30	30	25	100	7
CE	15	8	7	17	+1
	30	25	6	33	17
CNP	15	18	3	21	15
	30	20	5	24	17
CH	15	12	5	17	5
	30	28	13	46	17
CHE	15	27	16	42	+6
	30	48	27	100	3
CHNP	15	15	3	17	12
	30	35	8	40	29
CHNPE	15	9	9	15	2
	30	38	20	52	24
Control	15	+16		+16	+16
	30	+13		+13	+13

*Indicates mass increase.

have been summarized.[13] Although there is little variation in the expression of Col I and FN in mice and humans, the expression of Col II/III, Col IV, vitronectin, perlecan, and hyaluronan differed between the species.[13] The expression of laminins in mouse, rat, and guinea fowl was equivalent to the expression in human spleen. The differential expression of ECM molecules between species suggests that the suitability of choosing the appropriate digestive enzyme cocktail based on the species-specific histopathological characteristics of the spleen ECM may need to be considered for optimal digestion efficiency and accuracy.

Our results also show statistically significant mass reduction when spleens are treated with a dual intrasplenic injection and immersion approach *versus* extrasplenic enzyme digestion alone, validating our hypothesis that *in situ* enzymatic digestion via intraparenchymal and extrasplenic capsule dissolution leads to reduced spleen stiffness. These findings support the notion toward a novel alternative concept for specimen retrieval in the form of extracorporeal in-bag enzymatic spleen digestion to facilitate accelerated operative times and to reduce bag-related complications associated with manual morcellation.

Conclusion

The data in this preclinical study support murine spleen degradation when subjected to single or combination enzyme digestion. The injection of intraparenchymal enzyme solution combined with contained extrasplenic immersion offers mass reduction superior to that of immersion in enzyme solution alone. Specific splenic treatment with 50 mg/mL collagenase, 25 mg/mL hyaluronidase, and 12.5 mg/mL elastase at mean spleen to enzyme mass ratios of 6.5, 13, and 26, respectively, provides the best digestion within 30 min (mean 49%, maximum 100%). Regardless, the quantities of enzyme required for their use on a clinical scale represent a significant translational hurdle because of the prohibitive expensive cost for large-scale application. Despite this limitation, our study is the first to pave the way for evaluating alternative biologic specimen extraction methods for MIS splenectomy to replace mechanical extracorporeal morcellation. If a cost-saving approach could be tailored to larger specimens, then the findings observed in this study could be more usefully exploited as a new standard of contained morcellation and time-efficient spleen removal.

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Authors' contributions: Concept and design were done by J.A.S. and E.D.V. Acquisition, analysis, or interpretation of data were carried out by E.D.V., W.P.H., and J.A.S. Drafting of the article was done by E.D.V. and J.A.S. Critical revision of the article was done by E.D.V., W.P.H., and J.A.S. Statistical analysis was carried out by S.M., J.W., and W.P.H. Administrative, technical, or material support was given by J.A.S., W.H.L., and C.L.M. Study supervision was done by E.D.V., W.H.L., and J.A.S.

Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jss.2017.05.060.

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