Assessment of six different collagenase-based methods to isolate feline pancreatic islets

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Abstract

Isolation of pancreatic islets is necessary to study the molecular mechanisms underlying beta-cell demise in diabetic cats. Six collagenase-based methods of isolation were compared in 10 cat pancreata, including single and double course of collagenase, followed or not by Ficoll centrifugation or accutase, and collagenase plus accutase. Morphometric analysis was performed to measure the relative area of islet and exocrine tissue. Islet specific mRNA transcripts were quantified in isolates by real-time PCR. The single and double course of collagenase digestion was successful in each cat and provided similar islet-to-exocrine tissue ratio. Quantities of insulin mRNA did not differ between the two methods. However, on histological examination either method yielded only approximately 2% of pure islets. The other methods provided disrupted islets or insufficient samples in 1-7 cats. Although pancreas digestion with single and double course of collagenase was superior, further studies are needed to improve islet isolation in cats.
Title: Assessment of six different collagenase-based methods to isolate feline pancreatic islets

Running title: Isolation of pancreatic islets in cats

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Abstract

Isolation of pancreatic islets is necessary to study the molecular mechanisms underlying β-cell demise in diabetic cats. Six collagenase-based methods of isolation were compared in 10 cat pancreata, including single and double course of collagenase, followed or not by Ficoll centrifugation or accutase, and collagenase plus accutase. Morphometric analysis was performed to measure the relative area of islet and exocrine tissue. Islet specific mRNA transcripts were quantified in isolates by real-time PCR. The single and double course of collagenase digestion were successful in each cat and provided similar islet-to-exocrine tissue ratio. Quantities of insulin mRNA did not differ between the two methods. However, on histological examination either method yielded only approximately 2% of pure islets. The other methods provided disrupted islets or insufficient samples in 1-7 cats. Although pancreas digestion with single and double course of collagenase were superior, further studies are needed to improve islet isolation in cats.

Keywords: cat; accutase; β-cell; digestion; diabetes mellitus.
The hospital prevalence of diabetes mellitus has increased in the last decades in cats (Prahl et al., 2007). Evidence suggests that diabetes mellitus is more common because of a higher frequency of predisposing factors, such as obesity and reduced physical activity (Panciera et al., 1990; Scarlett et al., 1994). Overweight cats have markedly decreased insulin sensitivity and a compensatory increase in circulating insulin levels (Appleton et al., 2001). Insulin resistance imposes an excessive demand on pancreatic β-cells, which may lead to their exhaustion, impaired function and ultimately overt diabetes. In addition, lesions occurring in pancreatic islets, namely islet amyloidosis and partial loss of β-cells, are considered crucial to the development of the disease (Lutz and Rand, 1997; Appleton et al., 2001; Rand et al., 2004; Henson and O’Brien, 2006).

Studying the pathophysiology of feline diabetes is largely hampered by the lack of tools to characterize the molecular mechanisms through which glucose metabolism is disturbed, particularly in pancreatic islets and β-cells. Isolation of pancreatic islets is mandatory in order to characterize in detail the pathogenesis of islet amyloid deposition and β-cell dysfunction and loss in diabetic cats. Isolation of feline islets has been described in only one report (Maeno et al., 2006). In this study, collagenase digestion led to the recovery of isolated islets that were surrounded by residual exocrine pancreas. It was suggested that feline islets are difficult to separate completely from the exocrine pancreas because they are delimited by very little peri-islet matrix (Maeno et al., 2006). We also sought to isolate islets from healthy cats using the same collagenase-based protocol and achieved similar disappointing results (Zini et al., in press). The presence of residual exocrine tissue adjacent to isolated pancreatic islets makes it difficult to interpret the expression levels of islet target genes, in particular for transcripts that are also expressed in the exocrine pancreas. Genes that are up- or down-regulated in the residual acinar cells may artefactually modify the amount of transcripts
measured in the isolated islet preparations. Because islet isolation is technically demanding in cats (Maeno et al., 2006), information about the molecular mechanisms leading to impaired insulin secretion or loss of β-cells is currently unavailable in this species. In order to find a method yielding enhanced purity of isolated islets we devised and compared six different collagenase-based protocols of islet isolation in cats. As collagen is the major fibrous protein constituting the islet-exocrine pancreas interface, collagenases are most suited for islet isolation in humans and in experimental animals, including dogs, pigs and rodents (Ricordi et al., 1986; Kin et al., 2007; Ito et al., 2008). The collagenase-based method described by Maeno et al. (2006) was used as a reference. To improve the yield of pure islets, in addition to collagenase, the methods included Ficoll or accutase. Ficoll solutions allow gradient centrifugation of pancreatic islets and improve the retrieval of pure islets in humans and rodents, following collagenase digestion (Chun et al., 2008). Accutase is an enzymatic solution extracted from an invertebrate species containing collagenases and other proteases, and employed to isolate pancreatic islets or, more commonly, to separate β-cells after the islets have been isolated from human and rodent pancreas (Toyama et al., 2003).

The quality of isolated islets was assessed by means of morphometric analysis and real-time polymerase chain reaction (PCR) quantification of islet specific genes.
Materials and methods

Cats and isolation of pancreatic islets

Ten domestic cats that died or were euthanized due to severe illness were used. None of the cats was clinically diagnosed with pancreatitis or other pancreatic disease. Three cats had chronic renal failure, three had feline infectious peritonitis, two had lymphoma and one cat each had hypereosinophilic syndrome or idiopathic hypercalcemia. Cats were 1-19 years old (median age: 12 years). Seven cats were neutered males and three were spayed females. Nine cats were domestic shorthair, one was a Persian.

The pancreas was surgically excised under sterile conditions within 45 min from death. The isolated organ was immersed in Hank’s balanced salt solution (HBSS, Sigma-Aldrich, Buchs, Switzerland) and care was taken to remove surrounding fat tissue with sterile surgical scissors. The pancreas was weighed and processed according to the six protocols described below. A simplified outline of the isolation methods is provided in figure 1.

Method 1

Method 1 of islet isolation included collagenase and was performed as described by Maeno et al. in cats (Maeno et al., 2006). This method was used as a reference to compare with the newly developed protocols.

One portion of the pancreas, corresponding to 80% of the organ weight, was injected with chilled HBSS solution containing 1 unit/ml of collagenase (collagenase NB8, Serva Electrophoresis, Dübendorf, Switzerland) derived from the fermentation of Clostridium histolyticum. The tissue was injected across the pancreatic capsule using a 27G needle connected to a 10 ml syringe. The injected solution volume (ml) to pancreas weight (g) ratio was approximately 5:1 and the solution was injected in equal aliquots through several punctures. Injections were preferred because in the authors’ experience identification and
cannulation of the pancreatic duct is very difficult in cats and in most cases is not feasible. The injected tissue was placed in a Falcon tube containing 30 ml injection solution and incubated at 37 °C for 35-40 min. After digestion, the tissue was washed with ice-cold HBSS and filtered through a stainless steel screen with a 1 mm mesh. Half of the filtrate was used as described in method 4 (see below). The other half was placed in a new tube, filled up with 30 ml HBSS and allowed to sediment on ice for 5 min. Thereafter the supernatant was discarded and the sediment was washed 2 times at 5 min intervals with HBSS. The rinsed sediment was then split into two aliquots. One aliquot was transferred into a vial containing buffer RLT (Qiagen, Basel, Switzerland), frozen in liquid nitrogen and stored at −80 °C until further use. The second aliquot was placed in a vial containing 4% buffered formaldehyde and allowed to fix for 24 h. Then, the fixed tissue was pelleted on a paper towel and embedded into paraffin using the Cytoblock system (Thermo Shandon, Pittsburg, PA, USA) and standard embedding procedure.

Method 2

Method 2 was developed to improve the yield of pure islets (i.e., islets free of surrounding exocrine tissue) by exposing the digestate obtained in method 1 to a second course of collagenase digestion. The residual non-filtered tissue remaining on the steel screen from the preparation described in method 1 was immediately placed in a Falcon tube containing 30 ml HBSS with collagenase (1 unit/ml) for a second digestion at 37 °C for 20 min and then filtrated again. One fourth of the collected filtrate was washed with HBSS and the sediment was processed as in method 1.
Method 3

Method 3 was developed to include Ficoll solutions in the digestion protocol. The remaining
¾ of the filtrate collected in method 2 was placed in a Falcon tube and suspended in 20 ml of
the above collagenase enriched HBSS. Ten ml of Ficoll (GE Healthcare, Glattbrugg,
Switzerland) were slowly layered with a syringe under the 20 ml solution and centrifuged at
800 g for 20 min at 4 °C. Thereafter, only the digestate located at the interface between the
Ficoll and HBSS solution layers was carefully harvested. It was placed in a tube, washed with
HBSS and the sediment was processed as in method 1.

Method 4

Method 4 was developed to improve the yield of pure islets achieved in method 1 by
including accutase in the isolation protocol. Half of the digestate collected after filtration in
method 1 was used in the present method. It was suspended in 10 ml of accutase (Sigma-
Aldrich) and incubated at 37 °C for 10 min. The sediment was washed with HBSS, split into
two aliquots and processed as in method 1.

Method 5

Because accutase is used to isolate pancreatic islets but also to liberate single islet cells
(Toyama et al., 2003) and because detached cells or islet fragments may float in liquids, the
supernatant from the previous method was used. Supernatants may contain small islets, islet
fragments and single islet cells. The supernatant was centrifuged at 350 g for 10 min at room
temperature. The sediment was rinsed with HBSS, split into two aliquots and stored as in
method 1.
Method 6

Method 6 was developed to assess whether accutase may be more effective in islet isolation when the pancreas is simultaneously exposed to collagenase and accutase, rather than when accutase exposure follows collagenase digestion as in method 4.

The second part of the freshly excised pancreas, corresponding to 20% of the pancreas weight, was injected with a collagenase enriched HBSS (see method 1) mixed with accutase (Sigma-Aldrich). The volume ratio between collagenase enriched HBSS and accutase was 1:1. The tissue was injected as described above, placed in a Falcon tube containing the same amount of injection solution and incubated at 37 °C for 120 min. The digested tissue was washed with ice-cold HBSS and filtered through a stainless steel screen with a 1 mm mesh. The filtrate was placed in a new tube, washed with HBSS and processed as in method 1.

Histology and morphometric analysis

To identify the isolation protocol providing pancreatic islets with the least amount of residual acinar cells, sections of formalin-fixed paraffin-embedded digestate were prepared and subsequently immunohistochemically double-stained for insulin and glucagon using a polyclonal guinea pig antibody anti-swine insulin (Code A0564, Dako, Glostrup, Denmark) and a polyclonal rabbit antibody anti-human glucagon (Code A0565, Dako), as previously described (Zini et al., 2009). In brief, paraffin sections (3 μm) were deparaffinized in xylene and rehydrated through graded ethanol to water. The immunohistochemical reactions were performed using a commercially available detection kit (Dako REAL™ Detection system, peroxidase/AEC rabbit/mouse, Dako) according to the manufacturer's instructions. All steps were performed at room temperature in an automated device (Dako Autostainer, Dako). Primary antibody incubation conditions were 1:150 for 1 h for insulin and 1:200 for 1 h for glucagon. Double stains for insulin and glucagon were performed sequentially, first by
carrying on the complete procedure for glucagon including a final incubation step with the substrate AEC for 10 min and then carrying out the stain for insulin including a final incubation step with the substrate CAE (Code K3468, Dako) for 10 min. Non-counterstained sections were covered using KP-Tape (Klinipath, Duiven, The Netherlands).

The insulin- and glucagon-positive area relative to the cross-sectional area of the digestates was calculated using ImageJ software (http://rsb.info.nih.gov/ij/). At least 150 islets were included in the analysis. The percentage of pure islets relative to the total number of islets was calculated. Pictures were taken with an Olympus Vanox-S microscope (Olympus, Volketswil, Switzerland) and AxioVision digital processing system (Carl Zeiss, Oberkochem, Germany).

RNA isolation, reverse transcription and real-time PCR

Total RNA from pancreatic islets was extracted using RNeasy Mini Kit (Qiagen) following homogenisation of pancreatic digestates (30 mg). Possible genomic DNA contamination in islet-derived RNA samples was eliminated by including DNase-treatments (DNase-Free DNase Set, Qiagen). RNA was quantified spectrophotometrically and its quality was assessed by gel electrophoresis (i.e., evident bands of ribosomal RNA with minor background smear). cDNA was obtained from 1 μg samples of islet-derived RNA (Omniscript RT Kit, Qiagen) in the presence of 13 U of RNasin (Promega, Madison, WI, USA).

cDNA obtained from feline tissues was subjected to quantitative real-time PCR analysis using feline-specific intron-spanning oligonucleotides previously established for insulin and the housekeeping gene cyclophilin A (Zini et al., in press). Detection of insulin and cyclophilin A was achieved with the following sequences: insulin sense 5’-TTCGTCAACCAGCACCTGTG-3’, insulin antisense 5’-CACAGCATTGCCCTCCAGATG-3’, cyclophilin A sense 5’-CAAAGTTCCCAAGACAGAAGA-3’, and cyclophilin A antisense 5’-AGTGCCATTATGGCGTGTA-3’.
PCR reactions were prepared with 10 μL Power SYBR-Green Master Mix (Applied Biosystems, Foster city, CA, USA), a final concentration of 500 nM of each primer and 5 μL of template diluted 1:50 in a total reaction volume of 20 μL. Using the iCycler iQ sequence detection system (BioRad, Hercules, CA, USA), an initial denaturing step was performed for 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s, 60 °C for 30 s and 80 °C for 10 s, then 95 °C and 60 °C for 1 min each and 80 cycles of 10 s, starting from 60 °C with a 0.5 °C increase after each cycle. At the end of the programme, PCR product identity was confirmed by melting curve analysis.

Template-free controls and duplicates without reverse transcriptase were included in every amplification run. Parallel reactions were performed in triplicate for each cDNA sample. Target gene mRNA was quantified using the relative standard curve method. Standard curves were generated with serial dilutions of the purified specific PCR product previously obtained by conventional PCR with the same primers (Zini et al., *in press*). Insulin expression was normalised to the respective quantities of cyclophilin A.

**Statistical analysis**

Results are expressed as median and range. Morphometric data and mRNA transcript quantities were compared between isolation methods using the Wilcoxon matched pairs test or Friedman test followed by Dunn’s multiple comparison. To be considered suitable for analysis each protocol of islet isolation had to provide a sample for morphometry in all ten cats. The same criterion was applied to assess the suitability of each method for comparing mRNA transcript quantities. In addition, digestion methods were included in the investigation if islet morphology assessed on histological examination was preserved in at least 50% of the isolated islets. Islet morphology was considered not preserved if islets missed large fragments or islet cells were spread apart. We assume that islets disrupted through excessive digestion
contain damaged or metabolically stressed β-cells that may be less suitable for subsequent molecular studies. Statistical analysis was performed with GraphPad Prism version 4.0 (GraphPad Software, San Diego, CA, USA). In all cases, significance was set at $P < 0.05$. 
Results

Morphometric analysis of isolated islets

Methods 1, 2, 4 and 6 of islet isolation provided tissue digestates in all ten cats. Based on insulin and glucagon immunostaining and morphologic criteria, method 1 and 2 provided islets with preserved morphology in all cats. Method 4 and 6 provided islets that lacked large fragments or had islet cells spread apart in seven and in two cats, respectively (Fig. 2). Method 3 did not yield enough digestate for histological evaluation in one cat and method 5 in four. Upon morphometric analysis, method 1 and 2 provided similar ratios between islet and digestate cross-sectional area (Method 1, median: 0.03, range: 0.01-0.10; Method 2, median: 0.04, range: 0.01-0.06). Variation between cats was relatively high with both methods of islet isolation. For the same cat one method of islet isolation was generally accompanied by similar results with the other; as shown in Table 1, cats with the highest ratio between islet and exocrine tissue with one method generally had high ratio also with the other. Almost all islets retrieved with method 1 and 2 were surrounded by exocrine tissue and each isolation protocol yielded only very few pure islets (approximately 2%) (Fig. 3).

Insulin mRNA quantities in isolated islets

Methods 1, 2, 4 and 6 of islet isolation provided sufficient amount of pancreas digestate to isolate mRNA and quantify insulin transcripts in all cats. Method 3 did not yield enough digestate for real-time PCR analysis in one cat and method 5 in four. Quantities of insulin mRNA did not differ between method 1, 2, 4 and 6 (Fig. 4).
In the present study we aimed at improving isolation of pancreatic islets to facilitate future studies on the pathophysiology of diabetes. Ten cat pancreata were used to perform six collagenase-based digestion protocols, including a single course of collagenase (method 1) which corresponded to previously published method (Maeno et al., 2006), two courses of collagenase with or without Ficoll centrifugation (method 2 and 3), a course of collagenase followed by accutase with sediment and supernatant analysis (method 4 and 5), and concurrent digestion with collagenase and accutase (method 6).

Based on morphometric analysis, isolation of islets achieved with a double course of collagenase digestion provided similar results to the reference protocol with a single course of collagenase digestion (method 1) (Maeno et al., 2006). Islet morphology was preserved in each case but the large majority of material retrieved consisted of islets surrounded by residual exocrine pancreas suggesting that pancreatic digestion was far from optimal with both protocols. Digestion with collagenase followed by accutase with sediment analysis and concurrent digestion with collagenase and accutase yielded pancreatic islets that were excessively digested. With either method, more than 50% of the retrieved islets had lost a large number of cells. Even though the two digestion protocols disrupted most islets, the amount of exocrine tissue surrounding the islets was not reduced compared to methods 1 and 2. Because of excessive islet digestion the above methods incorporating accutase were excluded from comparison. The remaining methods, such as the two courses of collagenase digestion followed by centrifugation with Ficoll and the digestion with collagenase followed by accutase and supernatant analysis, did not yield enough tissue in some pancreata and were also excluded.

The specific type of enzymes may explain why protocols including accutase yielded pancreatic islets that were often disrupted. Accutase, in addition to collagenase, contains a
mixture of other proteases that contribute to enhance tissue dissociation (Wolters et al., 1992).

Proteases have been shown to decrease the islet yield in humans through excessive fragmentation and disintegration of pancreas (Bucher et al., 2004). It is therefore possible that this shortcoming accounted for the several islets without preserved morphology observed with protocols incorporating accutase.

Similar to morphometric data, insulin mRNA quantification was possible in all cats with the single and double course of collagenase digestion (method 1 and 2), with collagenase followed by accutase with sediment analysis (method 4), and with concurrent digestion with collagenase and accutase (method 6). Based on insulin transcript quantities, the four protocols performed equally. However, because the protocols employing accutase yielded excessively digested islets on histological examination, the single and double course of collagenase digestion were considered superior. Unfortunately, at the time of writing sets of primers for target expected to be specific for the exocrine pancreas (e.g., amylase) were not available in cats. This would have certainly helped assessing the amount of non-islet tissue obtained with the different isolation methods and evaluate further their effectiveness.

Even though the methods of islet isolation were performed according to standardized protocols, the ratio between pancreatic islet and exocrine tissue varied widely among cats. Nevertheless, in the same cat the ratio remained approximately similar when method 1 and 2 were employed, thus suggesting that individual differences were present. An explanation for this finding may be that the amount of collagen surrounding the islets differed from cat to cat. In humans it has been shown that increasing amount of collagen makes islet isolation less successful (Bedossa et al., 1989). However, we did not attempt to quantify peri-islet matrix in order to clarify the effect of collagen on the efficiency of islet isolation. We also did not look at inflammatory cells in the exocrine pancreas. The presence of pancreatitis in some cats may have rendered tissue digestion and islet isolation more difficult. In the authors’ opinion,
although increased collagen or inflammation may compromise the yield of retrieved islets, the
fact that all methods were compared in each single cat (i.e., paired analysis) excludes major
bias while preserving reliability of the results.

Another factor known to affect isolation efficiency is the use of collagenase obtained from
different batches. Indeed, enzyme potency may vary markedly among lots (Barnett et al.,
2005). To avoid this potential confounding factor, purchased collagenase and accutase
solutions originated from the same batches, respectively.

In the present study, pancreas digestion was performed by injecting the enzymatic solutions
with a needle across the pancreatic capsule. Several authors suggest that pancreas digestion
should rather be performed by injecting the enzymatic solutions through the pancreatic duct
(Kin et al., 2007). This technique may improve enzyme diffusion into pancreatic acini
followed by more selective digestion of the exocrine pancreas (Kin et al., 2007). Injection of
enzymatic solutions through the pancreatic duct was not performed in cats because the duct is
difficult to visualize and cannulate.

To improve the yield of pure islets additional enzymatic digestion solutions or advanced
methods of tissue isolation, such as laser-capture microdissection, may be considered. If these
methods will not improve islet isolation in cats, pancreatic digestion with single or double
course of collagenase may remain the alternative. Indeed, after pancreatic digestion has been
carried out with one of the two methods, pure islets can be manually selected under a
dissecting microscope with dithizone staining (Hansen et al., 1989). By using a larger part of
the pancreas than the one employed to perform this study, the yield of pure islets for
molecular investigation of β-cells most likely increases. Achieving enough pure islets is of
particular importance in case molecular and functional studies are concurrently planned. If
viable islets can be isolated in cats, the chance to perform in vitro studies would greatly
increase, thus allowing to investigating possible specific mechanisms of \( \beta \)-cell dysfunction and loss occurring in feline diabetes.

In summary, isolation of pancreatic islets by single and double course of collagenase digestion were the best methods of islet isolation in cats. However, because the large majority of retrieved islets were surrounded by residual exocrine pancreas, the average purity of isolates was not considered satisfactory. Further studies are clearly required to optimally isolate the islets and allow future investigation in feline \( \beta \)-cells.

**Acknowledgements**

We are grateful to Sabina Wunderlin for excellent technical assistance.

**Duality of interest**

The authors declare that there is no duality of interest associated with this manuscript.


Table 1. Individual values of calculated ratio between islet area to digestate cross-sectional area from method 1 and 2 of islet isolation.

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<thead>
<tr>
<th>Cat</th>
<th>Method 1</th>
<th>Method 2</th>
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<tr>
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<td>2</td>
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Figure legends

Figure 1. Outline of the six protocols (METHOD 1 to 6) adopted to isolate pancreatic islets in cats.

Figure 2. (A) Method 1 and (B) method 2 of islet isolation, immunohistochemical staining for insulin (dark-brown) and glucagon (red-brown). Most of the pancreatic islets are surrounded by undigested exocrine tissue. (C) Method 4 and (D) method 6 of islet isolation, immunohistochemical staining for insulin (dark-brown) and glucagon (red-brown). Large part of the pancreatic islets are lacking due to excessive tissue digestion.

Figure 3. (A) Method 1 and (B) method 2 of islet isolation, immunohistochemical staining for insulin (dark-brown) and glucagon (red-brown). Pure pancreatic islets not surrounded by exocrine tissue.

Figure 4. Quantities of insulin mRNA in cat pancreatic islets obtained with method 1, 2, 4 and 6 of islet isolation. Transcript quantities did not differ between methods. Values are expressed in relative units.
Figure 1

PANCREAS

collagenase (1st course)

METHOD 1
filtered
accutase
filtered
pellet
METHOD 4
supernatant
METHOD 5

METHOD 2
filtered

METHOD 3
Ficoll gradient

METHOD 6
filtered

collagenase + accutase

collagenase (2nd course)
Figure 3
Figure 4

[Diagram showing Insulin mRNA levels normalized to cyclophilin A for Method 1, Method 2, Method 4, and Method 6.]