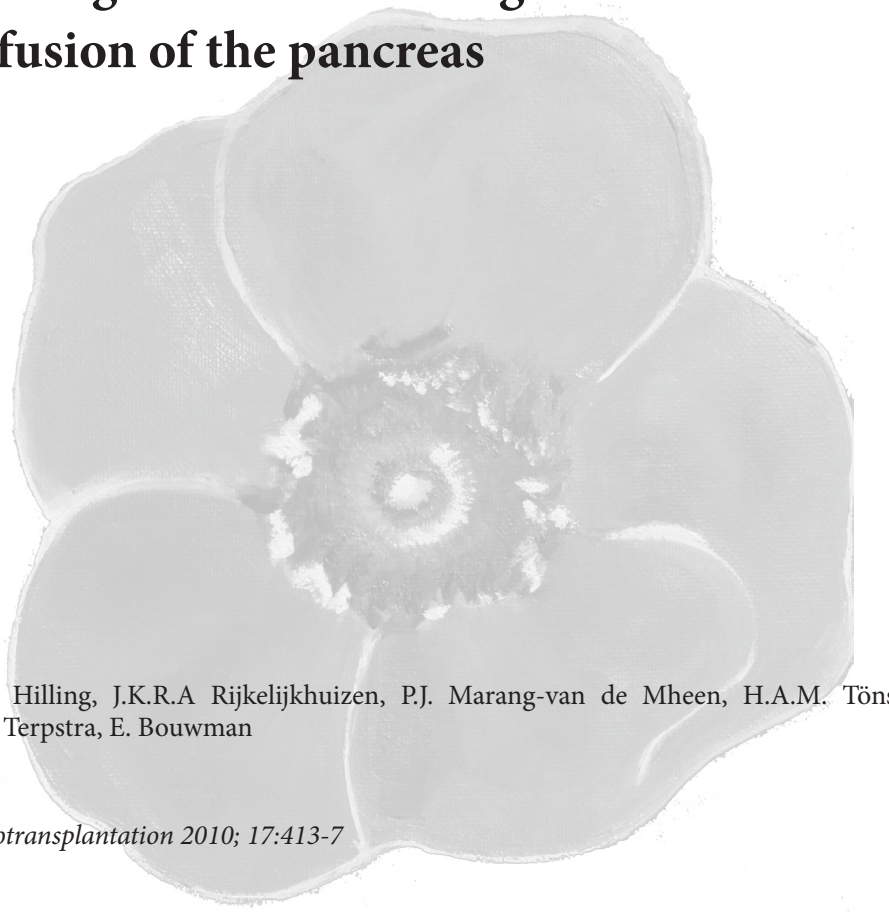
Chapter 8

Morphological changes of porcine islets of Langerhans after collagenase and HBSS infusion of the pancreas



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ABSTRACT

Background

A remarkable change in porcine islet morphology was observed after infusion of the pancreas with collagenase. The aim of the present study was to quantify these morphological changes and to assess whether these changes were due to the volume expansion caused by the collagenase entering the islet or the result of its digestive effects.

Methods and Materials

This study was performed in pancreata of 28 crossbred pigs. First, eight pancreata were intraductally injected with collagenase by a continuous controlled pressure of 180 mmHg. Pancreas samples before collagenase infusion were used as controls. All tissue samples, both before and after infusion, were stained with anti-insulin. To quantify the morphological change of the islets, the mean beta cell/endocrine content ratio of the infused and not-infused tissue samples was compared. In a second experiment, 20 pancreata were similarly assessed after intraductal injection with Hank's Balanced Salt Solution (HBSS).

Results

In both the collagenase- and HBSS-infused groups, mean beta cell/endocrine content ratio was lower than in the control samples. The observed decline in the beta cell/endocrine content ratio was not significantly different between collagenase- and HBSS-infused pancreata. This suggests that the lower beta cell/endocrine content ratio and thus the morphological change in the infused tissue samples is caused by volume expansion of the fluid entering the islet and that the digestive effect of collagenase plays no or only a minor role.

Conclusions

Morphological changes of islets are observed after infusion of pancreata with collagenase and HBSS, most likely caused by volume expansion due to fluid entering the islets.

INTRODUCTION

The pig is considered to be a potential alternative for human donors of islets of Langerhans. However, porcine islet isolation procedures have been shown to be notoriously difficult (1-4). Although procedures have been improved, porcine islet isolation is still associated with considerable loss of endocrine tissue. This indicates that not only the exocrine pancreas is affected by collagenase digestion, but that collagenase affects the islets as well. If we can find explanations for the loss of endocrine tissue, this may be a first step into finding a solution for this problem so that isolation procedures can be improved.

When studying histological characteristics of porcine pancreata in relation to islet isolation, a remarkable change in morphology of the islets was observed after infusion of the pancreas with collagenase. Previous studies have shown collagenase located within the islets after standard intraductal infusion (at a perfusion pressure of 180 mmHg) of collagenase in human and also at lower perfusion pressures (as low as 50 mmHg) in porcine pancreata (5,6). The observed morphological changes could therefore be a result of either volume expansion of collagenase entering in the islet, leading to disruption of cell-cell contacts or be the result of the digestive effect of collagenase, subsequently leading to islet fragmentation.

Other studies have reported on islet morphology after isolation (7,8). However, both studies have assessed the shape and size of islets after isolation, but did not perform a quantitative study of morphologic changes in islets after collagenase distension. Furthermore, it is not known whether the morphological changes are specific for collagenase or might also be observed for other fluids. If these are also shown for other fluids, this would suggest that the morphological changes are the result of volume expansion caused by fluid entering the islets.

The aim of the present study was to quantify the observed morphological changes after collagenase infusion. Furthermore, we aimed to discriminate whether these morphological changes are the result of volume expansion of collagenase entering in the islet or the result of the digestive effect of collagenase. To distinguish between these two hypotheses, a second series of experiments was performed in which pancreata distended with HBSS.

METHODS AND MATERIALS

Organ procurement and pancreatic sampling

A total sample of 28 pancreata of crossbred pigs (6-12 months) was harvested in a commercial slaughterhouse. All animals were killed by electric stunning and exsanguination. Warm ischemia times were between 20 to 30 min. After dissection

on ice, the pancreata were stored in cold (4°C) HBSS and transported on ice to the laboratory for further processing. Cold ischemia times were between 2 to 5 h, with most pancreata having a cold ischemia time around 3 h. Per pancreas, 1-cm biopsies of the duodenal part, splenic part and arm of the pancreas were taken directly before and some time after collagenase and HBSS infusion, immersed in Bouin's fixative, cleared and stored in ethanol 70%.

Collagenase and HBSS distension

Eight pancreata were distended with Liberase PI (Roche Applied Science, Manheim, Germany). Liberase PI was stored at a temperature of -20 °C and defrosted directly before injection. The pancreata were dissected free of surrounding fat and vessels in the laboratory. The main pancreatic duct was cannulated at the arm of the pancreas and intraductally injected with a solution of Liberase PI (0.5 mg/ml, solution temperature 4 °C at the start of infusion) in University of Wisconsin Solution (UW) by a continuously controlled pressure of 180 mmHg for 10 min. We have chosen 180 mmHg as perfusion pressure because it is used in standard infusion protocols. The syringe was removed before taking the biopsies. In a second experiment, 20 pancreata were intraductally injected with HBSS in the same way.

Histology

In paraffin-embedded sections of 4 μm, beta cells were stained with a polyclonal peroxidase-labeled guinea pig anti-porcine insulin antibody (Zymed, Invitrogen, Carlsbad, CA, USA) at a dilution of 1:200 overnight and visualized (brown colored staining) with 3,3'-diaminobencidine (DAB) dissolved in Tris/HCl (0.05 M) with 15 μl H2O2. The specificity of the antibody was confirmed by a negative control. Infused and not-infused tissue samples were stained in the same way.

Microscopic evaluation

An average of 75, randomly selected, islets per tissue sample were assessed. As is generally practiced, islets smaller than 50 mm were not included. To quantify the beta cell content (Fig. 1A,D), the brown stained area was quantified using the Zeiss KS400 image analysis system (Zeiss-Vision, Germany). The Zeiss KS-400 image analysis system quantified the brown-stained area per field, by calculating the percentage of brown staining in each field. Image J (freeware image processing tool) was used to manually delineate every islet used in the assessment of the beta cell content (Fig. 1B,E). The islets were independently delineated by two observers. The observers were blinded for the category of the tissue samples (collagenase/HBSS-infused or not-infused tissue samples) when delineating the islets.

In human islets (consisting of alpha, beta, delta and PP cells), beta cells represent the majority of the endocrine content. Because porcine islets have comparable

cytoarchitecture as human islets, where the outermost cells are commonly beta cells (9-11), the area inside the surrounding line was considered to represent the endocrine content and colored black using Image J (Fig 1C,F). This black colored area was also quantified using the Zeiss KS400 image analysis system by calculating the percentage of black staining in each field. The ratio between the beta cell and endocrine content was calculated by dividing both percentages (of brown and black staining) in both the infused and not-infused tissue samples. Per tissue sample an average beta cell/endocrine content ratio was calculated.

To quantify alterations in islet morphology, the average beta cell/endocrine content ratio of not-infused and infused tissue samples of the same pancreas were compared. An in- or decline in beta cell/endocrine content ratio after infusion would implicate a change in islet morphology. For example, the greater the decline in beta cell/endocrine content ratio, the greater the morphologic change after collagenase/HBSS infusion would be.

Data analysis

Statistical analysis was performed using SPSS 16.0 (IBM Corporation, Somers, NY, USA) statistical software. After visual inspection of the data, these were found to be normally distributed so that parametric tests were used. Because the beta cell/endocrine content ratio after infusion is related to the beta cell/endocrine content ratio before infusion, these ratios were compared for every individual pancreas using paired student t tests in both HBSS- and collagenase-infused pancreata. To assess the influence of the infused fluid, we calculated the difference between the beta cell/endocrine content before and after infusion for every individual pancreas and consequently compared the average difference between collagenase- and HBSS-infused pancreata using independent student t tests Results are presented as mean ± SD.

RESULTS

In a pilot experiment, three observers (DEH, EB and a medical student) independently assessed infused and not-infused tissue samples of eight collagenase-distended pancreata. The tissue samples were blinded in such a way that the observers were unable to distinguish from the outside whether the samples were infused or not-infused tissue samples. All observers could discriminate the infused and not-infused tissue samples with 100% accuracy, based on the substantial morphological differences observed in the histological structure of the islets and exocrine tissue.

No significant difference was found between the two observers in the quantification of the endocrine content ($p = 0.90$). The average of the two observers was therefore used in further assessments.

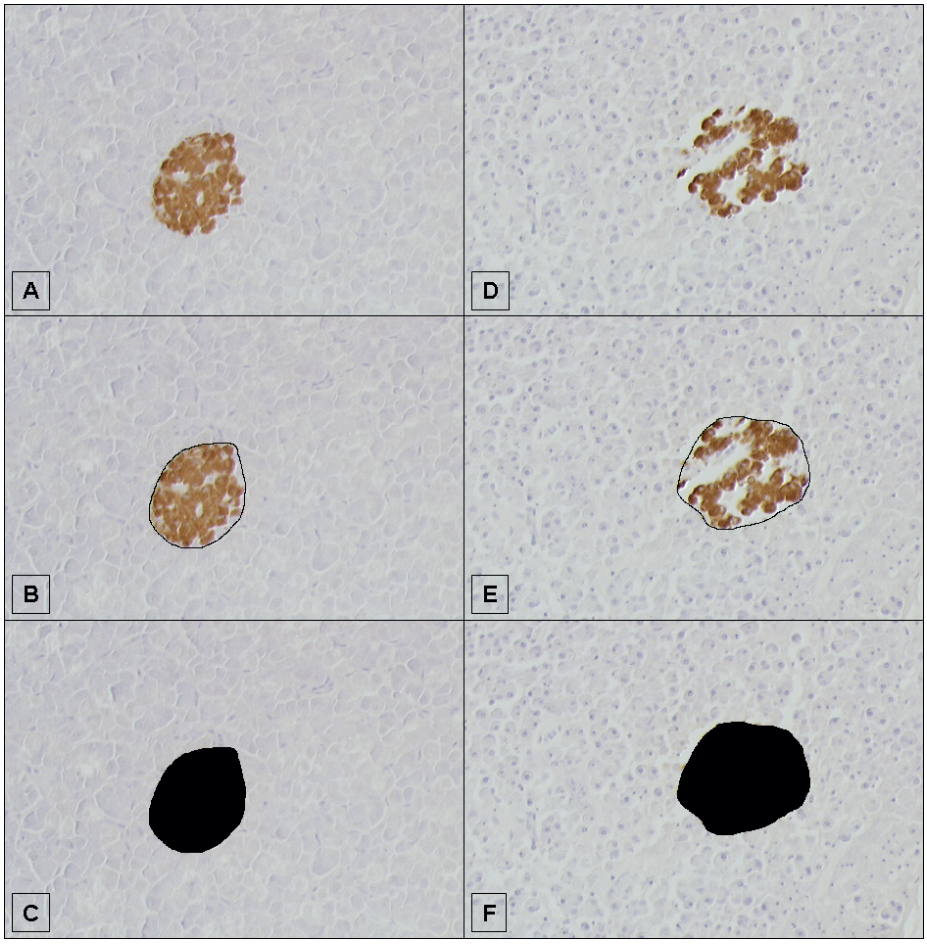

Figure 1: Calculation of the beta cell/endocrine content ratio
(A) Islet of a not-infused tissue sample. The brown stained (beta cell) area was quantified usinag an image analysis system. To quantify the endocrine content, every islet used in the assessment of the beta cell content, was surrounded by a manually drawn line (B). The area inside the surrounding line was considered to represent the endocrine content (C). The ratio between the beta cell and endocrine content was calculated: area A/area C = ratio not-infused.
(D) Islet of the same pancreas, infused with HBSS. The brown-stained (beta cell) area and endocrine content were determined in similar fashion as in the not-infused tissue sample (E, F). The ratio between the beta cell and endocrine content was calculated: area D/area F = ratio infused.
To quantify the morphological change after infusion, the ratios of the not-infused and infused tissue samples were compared.

In eight collagenase-infused pancreata, mean beta cell/endocrine content ratio (± SD) in the not-infused tissue samples was 0.44 ± 0.08. After infusion this ratio was significantly lower: 0.34 ± 0.07 ($t = 2.545$, $p = 0.04$) (Fig. 2). In 20 HBSS-infused pancreata, mean beta cell/endocrine content ratio in the not-infused tissue samples was 0.49 ± 0.09. After infusion this ratio was significantly lower: 0.38 ± 0.09 (t =

3.795, p = 0.001) (Fig. 2). No significant differences in beta cell/endocrine content ratios were found between different parts of the pancreas in both groups (data not shown). Furthermore, we found no significant differences between the beta cell mass or endocrine content before and after infusion of the pancreas with either collagenase or HBSS (data not shown).

To assess the influence of the infused fluid, the difference between the beta cell/endocrine content ratio before and after infusion was compared between collagenase- and HBSS-infused pancreata. In collagenase-infused pancreata the mean decline of the beta cell/endocrine content ratio after infusion was 0.10 ± 0.11. In HBSS-infused pancreata, the mean decline of the beta cell/endocrine content ratio after infusion was 0.11 ± 0.13 which is similar to the decline in collagenase-infused pancreata (t = 0.316, p = 0.76). This suggests that the beta cell/endocrine content ratio in the infused tissue samples is lower regardless of the infused fluid, so that it seems likely that this lower ratio is caused by volume expansion of the fluid entering in the islet. Because the lower

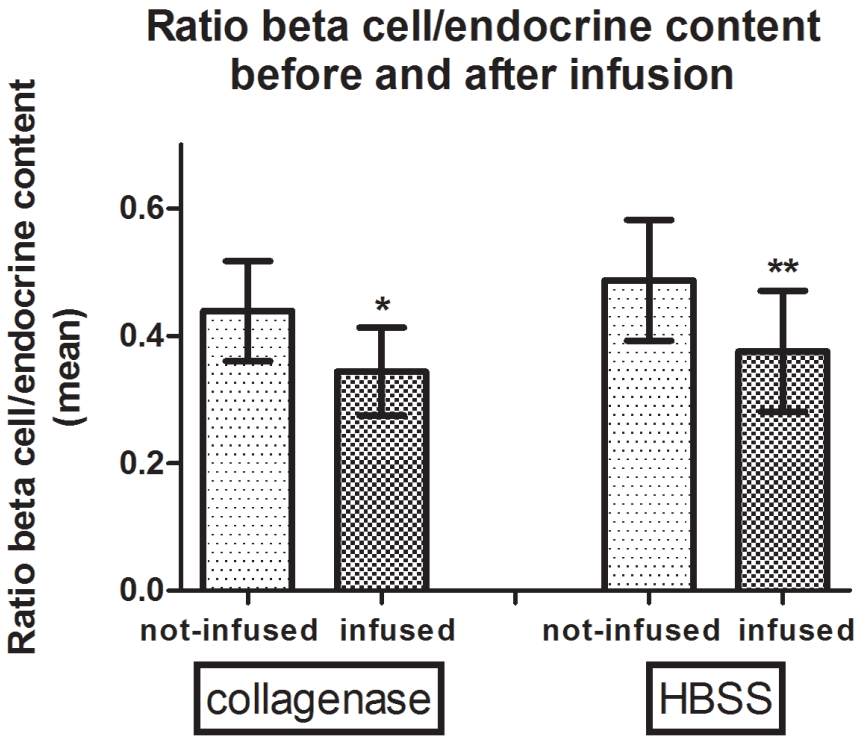

Figure 2: Ratio beta cell/endocrine content before and after collagenase and HBSS infusion
The bars represent the mean and the error-bars the standard deviation.
In the collagenase infused pancreata, mean beta cell/endocrine content ratio in the not-infused tissue samples was 0.44 ± 0.08. After infusion this ratio was significantly lower 0.34 ± 0.07, * p = 0.04
In the HBSS infused pancreata, mean beta cell/endocrine content ratio in the not-infused tissue samples was 0.49 ± 0.09. After infusion this ratio was significantly lower: 0.38 ± 0.09, ** p = 0.001.

ratio is not specific for collagenase, it seems that the digestive effect of collagenase plays no or only a minor role. After infusion, the weight of the pancreas was found to be 2.8 times higher than the weight before infusion, in both groups. This supports the hypothesis that there is an overall swelling of the organ, because the fraction endocrine content is too small to account for this increase.

DISCUSSION

Ductal injection of collagenase has been shown the technique to produce the highest isolation yields. However, even when collagenase is delivered to the pancreas in this way, there is still a considerable loss of endocrine tissue. As a first step in finding explanations, this study showed that islets undergo a morphological change during an islet isolation procedure currently used in most porcine and human islet isolations. The observed decline in the beta cell/endocrine content ratio was not specific for collagenase, but also shown for HBSS, so the morphological changes seem most likely to be due to volume expansion. These results are supported by previous studies by Johnson et al. (6) and Cross et al. (5) which showed the presence of collagenase within the islets of porcine and human pancreata after collagenase distension. The present study adds that other fluids may have the same effect.

In the present study, the endocrine content was determined by manual delineation of the islets used in the assessment of the beta cell content. Because the assessment of the endocrine content could potentially have been influenced by the observer performing the delineation, this could have influenced our results. However, because the delineation was performed by two observers and no significant difference between these observers was found, our study results do not seem greatly affected by this.

Peak activity of collagenase can be found at 37°C. In our study, we did not heat the enzyme to this temperature, as we would have performed when isolating the islets. This may have underestimated the potential effect of collagenase. However, because the biopsies after infusion were taken some time after infusion of the pancreas and the pancreas was not chilled during infusion, the temperature of the enzyme at that point would most likely lie close to room temperature. At this temperature collagenase already has considerable activity and could have influenced islet morphology. However, we cannot exclude that, when heated to 37°C, the digesting effect of collagenase does influence islet morphology.

We have compared HBSS-infused pancreata with pancreata infused with collagenase dissolved in UW. This could potentially have influenced our results since the enzyme-free solution is different than the solution in which the enzyme is dissolved. However, since UW is commonly used in porcine islet isolation procedures and is not

known to have a negative influence on porcine isolation outcome, our results do not seem greatly affected by this.

Moreover, we only have assessed the change in morphology of the islets some time after infusion of the pancreas with collagenase and HBSS and did not assess the effect of the rest of the isolation procedure on islet morphology, further research is necessary to assess whether these effects remain over time or are resolved during further processing of the pancreas.

The results of this study may provide part of the explanation for the loss of endocrine tissue during isolation procedures. The volume expansion caused by collagenase entering into the islet could lead to disruption of cell-cell contacts, leading to islet fragmentation and eventually reduced islet yields. Also the pressure of the infusion of the fluid could lead to disruption of cell-cell contacts. Another explanation could be a change in islet density. After digestion of the pancreas, separation of exocrine and endocrine tissue by a gradient is based on a difference in density, with the exocrine having a higher density than the endocrine tissue. When collagenase enters the islet, this could change the density of the endocrine tissue and lead to a smaller difference in density between the endocrine and exocrine tissue. It would consequently be more difficult to separate these two fractions by a gradient leading to a reduction in isolation outcome.

Besides lower yields, the volume expansion caused by collagenase could also play a role in impaired function after isolation, simply as a result of mechanical pressure from ductal infusion on the membranes of acinar cells leading to fluid entering vasculature and islets. This is supported by Dufrane et al. (11) who showed that large, well-structured porcine islets with central capillaries possessed a better potential for cellular engraftement than disrupted islets. In addition, other studies have shown that disruption of the microanatomy of the islets results in alteration of insulin secretory responses (12) and that glucose hemostasis not only depends on the number and integrity of beta cells, but also on their interaction with neighboring beta and non-beta cells (13).

In conclusion, morphological changes of islets after collagenase infusion seem to depend on volume expansion caused by fluid entering the islets. This could potentially lead to islet fragmentation, resulting in reduced islet isolation outcome and impaired function. If future research finds a way to inject collagenase without the fluid entering the islets, this may result in a considerable improvement of isolation yields.

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