Improving the use of donor organs in pancreas and islet of Langerhans transplantation

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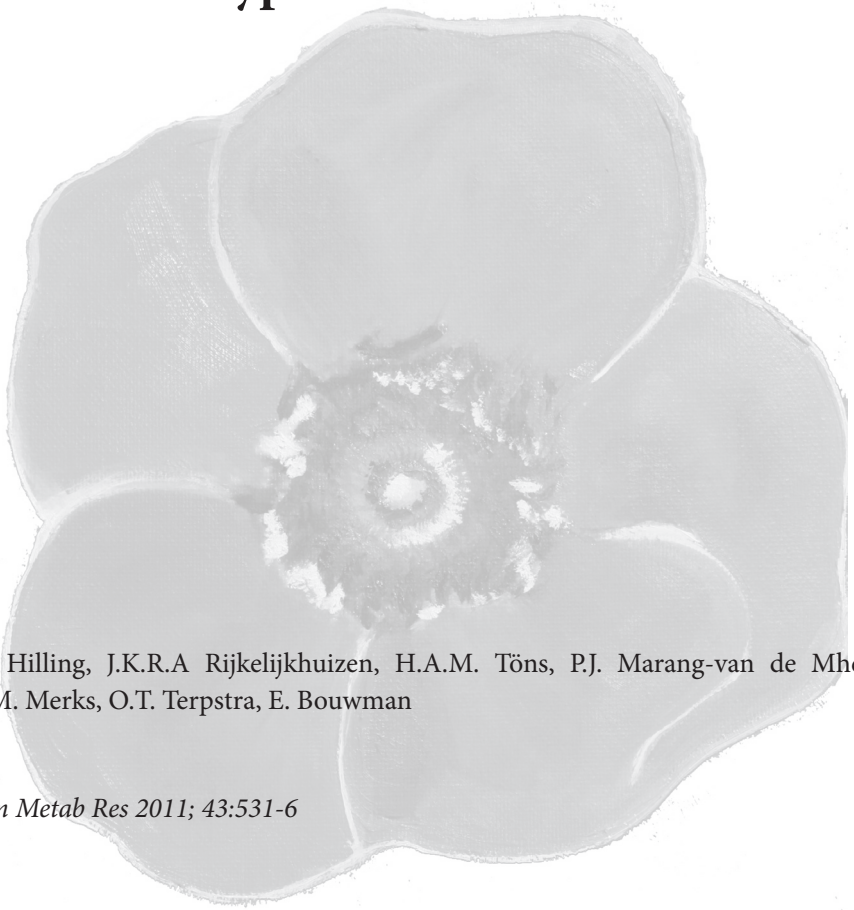
Part III

Porcine islet transplantation

Chapter 6

Reduced porcine islet isolation yield in the presence of hyperemic islets



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ABSTRACT

Background

When studying histological characteristics of porcine pancreata in relation to islet isolation, a remarkably high number of hyperemic islets (HIs) was encountered. The abnormalities observed in these HIs ranged from a single dilated vessel to hemorrhages extending into the surrounding exocrine tissue. The aim of the present study was to compare pancreata with and without HIs on islet isolation outcomes.

Methods and Materials

This study involved a histological examination of 143 purebred (74 juvenile and 69 adult) and 47 crossbred (only juvenile) porcine pancreata. Islet isolation was performed in 48 purebred adult pigs and in 25 crossbred pigs. Tissue samples were stained with Aldehyde Fuchsine. The presence of HIs was scored semi-quantitatively (HI-, HI+).

Results

We observed HIs in 48% of the purebred and in 68% of the crossbred pigs. However, only 3.3 ± 3.1% and 3.1 ± 4.7% of all assessed islets was hyperemic in HI+ pancreata in purebred and crossbred pigs, respectively. In both groups, significantly higher endocrine cell mass was found in the HI+ pancreata ($p < 0.01$). When the higher endocrine cell mass was taken into account, we found significantly lower yields in the HI+ pancreata in both purebred and crossbred pigs ($p = 0.03$ in both groups).

Conclusions

The presence of HIs occurs frequently in porcine donor-pancreata and is associated with reduced isolation outcomes.

INTRODUCTION

Much research has been conducted to optimize the outcome of porcine islet isolation. The amount of endocrine tissue present in a specific pancreas is undoubtedly an important factor in determining the islet isolation outcome. However, a high endocrine cell mass does not ensure a high isolation yield. Other factors, such as collagen and other matrix elements are thought to play a role (1-6). It is presently unclear to what extent such factors are dependent on breed, sex and age.

When studying histological characteristics of the porcine pancreas in relation to islet isolation, a remarkably high number of hyperemic islets (HIs) was encountered, a phenomenon that has been described only anecdotally, but not been studied in detail before (7-11). The abnormalities observed in these HIs ranged from a single dilated vessel through multiple widely dilated vessels to hemorrhages extending into the surrounding exocrine tissue. We have previously reported our study of human donor-pancreata where we found that the presence of HIs is associated with reduced isolation outcomes (12).

The aim of the present study was to assess whether this is a general phenomenon also present in porcine pancreata, and to determine the frequency of HIs in porcine pancreata compared to human pancreata. Furthermore, we studied the occurrence of HIs in relation to the amount of endocrine tissue in situ and in relation to the outcome of islet isolation to assess whether a similar relation was found as in human pancreata. Because external factors (e.g. breed, nutrition, age and transportation) could potentially have a confounding effect on islet isolation outcome, we assessed 2 distinctly different groups: purebred and crossbred pigs.

MATERIALS AND METHODS

Organ Procurement and Pancreatic Sampling

A total of 143 purebred and 47 crossbred porcine pancreata were harvested in different commercial slaughterhouses. The purebred population consisted of a juvenile group (6 – 12 months) of 74 animals and involved 8 different breeds (Great Yorkshire n = 18, Dutch Landrace n = 25, Norwegian Landrace n = 3, Large White n = 11, Hampshire n = 2, Finnish Landrace n = 7, Duroc n = 8) The adult purebred group (12 – 78 months) consisted of 69 animals and involved 8 different breeds (Great Yorkshire n = 14, Dutch Landrace n = 17, Norwegian Landrace n = 6, Large White n = 7, Hampshire n = 3, Finnish Landrace n = 10, Duroc n = 10). The crossbred group consisted of only juvenile animals (6 – 12 months) and were a cross of 2 or 3 different breeds.

All animals were killed by electric stunning and exsanguination. Warm ischemia times were between 20 – 30 min. After dissection, the pancreata were stored in cold

(4°C) Hank's Balanced Salt Solution (HBSS) and transported on ice to the laboratory for further processing. Cold ischemia times were between 2 – 5 h. Biopsies were taken from the splenic, duodenal and connecting lobes, immersed in Bouin's fixative, cleared and stored in ethanol 70%.

Histology

From all pancreata, paraffin-embedded sections of 4 μm were stained using the Aldehyde Fuchsine-Halmi (AF) technique: Rehydration, 90 seconds 2.5% potassium permanganate, 2' distilled water, 2' 1% oxalic acid, 10' running water, 1' distilled water, 15' AF, 3' 90% ethanol (2 times), 1' 70% ethanol, 1' 50% ethanol, 5' aquadest, 10-60 s Halmi, 10 s 0.2% acetic acid, 2' 100% ethanol (2 times), 5' xylene (2 times). Sections were embedded in malinol (Chroma-Gesellschaft, Köngen, Norway).

Microscopic evaluation

The same method was used as reported previously for human pancreata [12]. We have assessed the relation between the presence of HIs and endocrine cell mass, because pancreata with higher endocrine cell mass could produce higher yields simply because there is more endocrine cell mass to begin with. This could potentially confound our results when assessing the relation between the presence of HIs and isolation outcome.

Islet sizes were determined in each pancreas using a calibrated grid and grouped into 8 categories: >50-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400 and >400 μm diameter. As is generally practiced in the endocrine quantification in islet isolation procedures, islets smaller than 50 μm were not included because of their neglectable contribution to the total endocrine cell mass. For determination of the endocrine cell mass and the islet numbers, all islets in a tissue area of 1 cm^2 per slide were assessed. The endocrine area density was determined by summation of all calculated (πr^2) islet areas and expressed as percentage of the total area. According to the principle of Delesse (area density = volume density) and the assumption that islets are spherical, the determined endocrine area represents the endocrine cell mass. Assuming that the islet density does not significantly differ throughout the pancreas, the endocrine cell mass that is found in 1 cm^2 tissue represents the percentage of endocrine tissue in the pancreas.

Islets described as hyperemic in this study ranged from islets showing only a single slightly dilated vessel to hemorrhages extending into the surrounding exocrine tissue (Fig.1). The presence of HIs was scored semi-quantitatively (HI-, HI+) as done in our study of human pancreata (12). HI- pancreata contained none and HI+ pancreata at least one hyperemic islet.

To assess the influence of the severity of the hyperemia, the HI+ pancreata were subdivided into HI+ and HI++ pancreata, also on a semi-quantitative basis. HI++ pancreata were more severely affected than the HI+ pancreata. Because of the

enormous diversity in the occurrence of hyperemic islets both the amount of affected islets and the severity of hyperemia in the tissue sample were considered. For example, a tissue sample with 2 severely affected islets (with hemorrhages extending into the surrounding exocrine tissue, as shown in Fig. 1F) was scored as ++, while another tissue sample with 3 only slightly affected islets (with 1 dilated vessel, as shown in Fig. 1B) was scored as +.

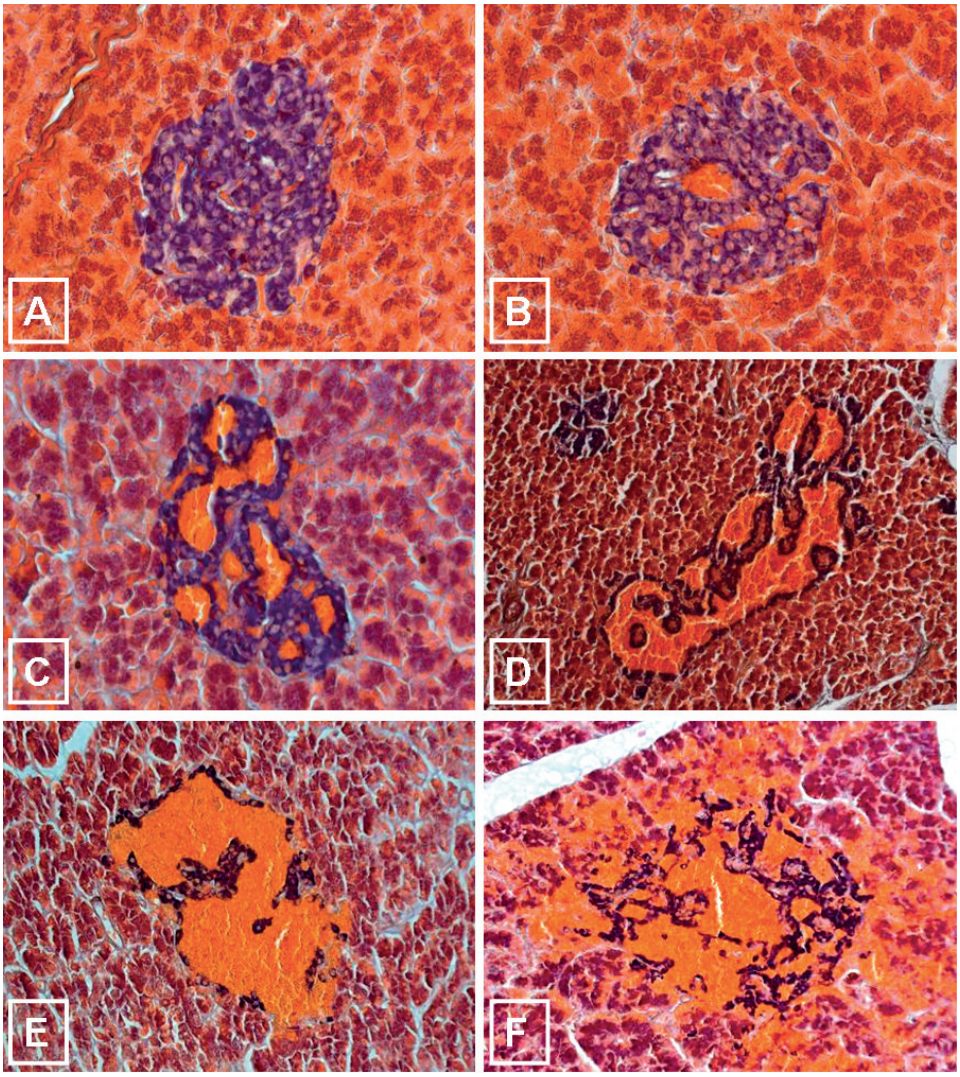

Figure 1: Histology of porcine pancreata (AF staining) showing different stages of hyperemic islets.
(A) Normal islet. (B) Islet with a single dilated vessel. (C) Islet with multiple dilated vessels. (D) Swollen islet with vastly dilated vessels and only a rim of islet cells left. (E) As in picture D, with several small hemorrhages breaking through the rim. (F) Grossly enlarged and distorted islet with multiple hemorrhages penetrating deeply into the surrounding exocrine tissue.

The pancreata were independently allocated to the different categories by 2 observers. The observers were blinded for the isolation outcome when allocating the pancreata to the different categories. Any differences between observers in the allocation of the pancreata, were resolved by discussion.

Average beta cell/islet area ratio per islet and total number of islets

To assess possible swelling of the islets, the average beta cell/islet area ratio per islet was compared in HI+ and HI- pancreata, consistent with the method used previously (12). This was done in a smaller sample of 44 randomly selected pancreata (22 HI+ and 22 HI-). Furthermore, the total number of islets was assessed by counting all islets, including islets < 50 μm, in an area of 1 cm^2 tissue in these pancreata.

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 with 3,3'-diaminobencidine (DAB) dissolved in Tris/HCl (0.05 M) with 15 μl H_2O_2.

An average of 75 islets per tissue sample was assessed. To quantify the beta cell area, the brown stained area was quantified using the Zeiss KS400 image analysis system (Zeiss-Vision, Germany). Only islets larger than 50 μm in diameter were included. To quantify the islet area, every islet used in the assessment of the beta cell area, was surrounded by a drawn line, manually delineated with a pen tablet using ImageJ (freeware image processing tool). In human islets, beta cells represent 50-60% of the endocrine cell mass. Because alpha, beta and delta cells have been shown to appear scattered throughout the islets and porcine islets have been shown to have similar cytoarchitecture as human islets (13,14), the area inside the surrounding line was considered to represent the islet area. This area was also quantified using the Zeiss KS400 image analysis system. To quantify a possible swelling of the islets, the ratio between the beta cell and islet area was calculated. The greater the swelling of the islets due to vasodilatation or edema, the lower the beta cell/islet area ratio would be.

Islet isolation

In the purebred group, islet isolation was performed in 48 adult animals. In the crossbred group, islet isolation was performed in 25 animals. The pancreata were cut clean in the laboratory. The arm of the pancreas was intraductally injected with a solution of Liberase PI (0.5 mg/ml, Roche Applied Science, Germany) in University of Wisconsin solution (UW). The distended tissue was cut into pieces and incubated in HBSS at 37°C for 20 – 30 min. The digested tissue was placed in a beaker on ice, which was manually shaken to gently dissociate and dilute the tissue. Subsequently, the tissue was poured over a filter with 1000 μm mesh and washed in cold HBSS for 3 times. Islet samples of 25 μl were stained with an equal volume of Dithizone solution (DTZ, diphenylthiocarbazone, Sigma, Steinheim, Germany), freshly prepared by the addition

of 5% DTZ stock-solution (13 mM DTZ dissolved in 91.2% ethanol supplemented with 1.2% NH_4OH) to HBSS. Islet numbers, expressed as islet equivalents (IE, representing islets of 150 μm diameter), were determined in duplicate.

Data analysis

Statistical analysis was performed using SPSS 16.0 statistical software. HI- and HI+ pancreata were compared on endocrine cell mass, number of islets, isolation outcome and the average beta cell/islet area ratio per islet using student *t*-tests. HI-, HI+ and HI++ pancreata were compared to assess the influence of the severity of hyperemia on the yield/islet area ratio using one-way analysis of variance. Post hoc Bonferroni's multiple comparisons tests were performed to determine which groups differed from each other. To assess the influence of the percentage HIs in a pancreas on isolation outcome, linear regression analysis was performed with yield/islet area ratio as dependent and percentage HIs as independent variable. P values ≤ 0.05 were considered to be statistically significant.

RESULTS

Presence of HIs

Histological analysis showed that 48% of the pancreata in the purebred group contained at least one HI and were scored as HI+. HIs were found more frequently in juvenile than in adult purebred pigs (64% vs. 32%, $p < 0.001$). In the crossbred group, HIs were found in 68% of the pancreata. In purebred HI+ pancreata, 3.3 ± 3.1% of all assessed islets was hyperemic. In crossbred pancreata 3.1 ± 4.7% of all assessed islets in HI+ pancreata was histological abnormal.

We assessed the presence of HIs in different parts of both purebred and crossbred pancreata. There were no differences in the occurrence of HIs in either the splenic, duodenal or connecting lobes in both groups. We also found no significant differences in the occurrence of HIs between the different breeds (results not shown).

An experienced pathologist assessed a random selection of 10 HI+ and HI- pancreata. The exocrine tissue and the non-hyperemic islets appeared to be visually normal in both groups of pancreata. In the HI+ pancreata no macrophages or iron deposits were seen and no evidence for an ongoing chronic process was found. Beside the presence of HIs, no differences were seen between the HI+ and HI- pancreata.

Endocrine cell mass in relation to HIs

In both the purebred and crossbred group, significantly higher endocrine cell mass was found in the HI+ pancreata ($p = 0.006$ and $p < 0.001$, respectively) (Fig. 2A,C). HI+ pancreata were also found to contain significantly higher number of islets in 1

cm^2 tissue ($p = 0.009$ and $p < 0.001$, respectively) (Fig. 2B,D). To assess the influence of the severity of the hyperemia on the endocrine cell mass and number of islets, the HI+ pancreata were subdivided into HI+ and HI++ pancreata. One-way analysis of variance showed that the mean endocrine cell mass differed between the groups in both purebred and crossbred pigs ($p = 0.001$ and $p < 0.001$, respectively). In both purebred and crossbred pigs, Bonferroni's test revealed that the endocrine cell mass was significantly higher in the HI++ group compared to the HI- ($p < 0.001$ and $p < 0.001$, respectively) and HI+ ($p = 0.02$ and $p = 0.001$, respectively) groups. One-way analysis of variance showed that the mean number of islets differed between the groups in both purebred and crossbred pigs ($p < 0.001$ and $p < 0.001$, respectively). In both purebred and crossbred pigs, Bonferroni's test revealed that the number of islets was significantly higher in the HI++ group compared to the HI- ($p < 0.001$ and $p < 0.001$, respectively) and HI+ ($p = 0.003$ and $p = 0.007$, respectively) groups.

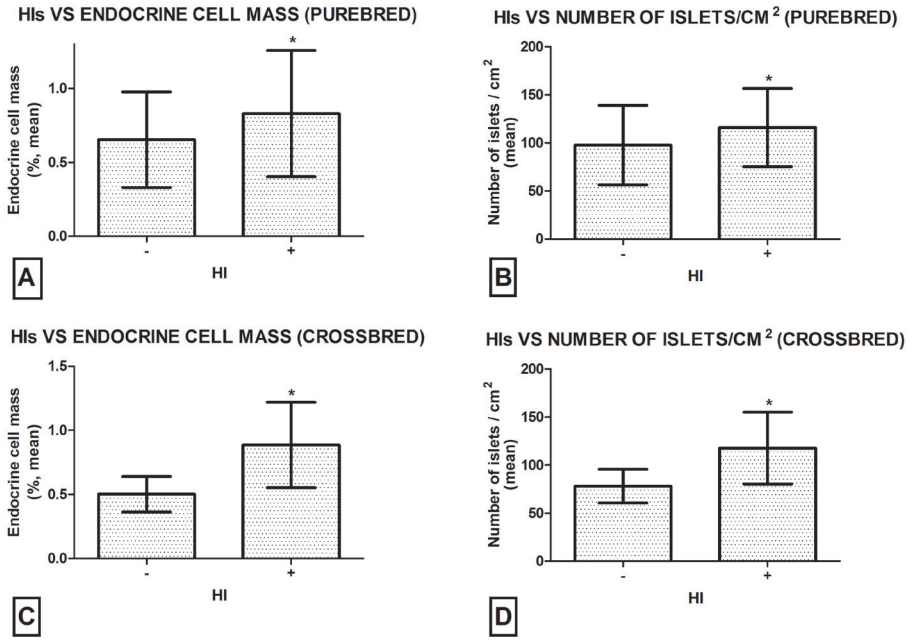

Figure 2: The endocrine cell mass and number of islets in relation to hyperemic islets.
The bars represent the mean endocrine cell mass (A,C) and the mean number of islets per cm2 (B,D). The error bars represent the standard deviation.
(A,B) Pancreata from purebred pigs. (A) Mean endocrine cell mass in HI- pancreata was 0.65 ± 0.32%. In HI+ pancreata this was 0.83 ± 0.43% *p = 0.006 (B) Mean number of islets per cm2 was 97.8 ± 41.2 in HI- and 116.0 ± 40.7 in HI+ pancreata *p = 0.009
(C,D) Pancreata from crossbred pigs. (C) Mean endocrine cell mass in HI- pancreata was 0.50 ± 0.14%. In HI+ pancreata this was 0.89 ± 0.33% *p < 0.001 (D) Mean number of islets per cm2 was 78.2 ± 17.5 in HI- and 117.7 ± 37.3 in HI+ pancreata *p < 0.001.

When we subdivided the purebred group into juvenile and adult pigs and assessed them separately, similar results were observed (data not shown).

These results suggest that the higher endocrine cell mass in the affected pancreata is associated with a higher number of the islets. The endocrine cell mass in HI+ pancreata was found to be 1.7 and 1.3 times higher compared to HI- pancreata in both purebred and crossbred pigs. It is not immediately obvious how this difference can be explained. Although the increased endocrine volume is paralleled by an increase in islet numbers, a straightforward comparison is confounded by the fact that only islets of greater than 50 μm were counted. When the underlying cause of the increased endocrine cell mass would be expansion of the individual islets, then a substantial number of islets smaller than 50 μm might cross the 50 μm limit and enlarge the total pool of countable islets. Alternatively, the most affected pancreata might simply have 1.7 and 1.3 time greater islet numbers. To elucidate this, we have assessed the average beta cell/islet area ratio per islet and furthermore counted all islets, including islets < 50 μm, in a limited series of 22 HI+ and 22 HI- pancreata. Furthermore, to assess and quantify possible swelling of the islets, we determined the average beta cell/islet area ratio per islet. The mean beta cell/islet area ratio in the HI- group was 0.57%, which was significantly higher ($p = 0.004$) than in the HI+ pancreata where this was 0.52%. No significant difference was found between the numbers of islets/cm^2 in HI- and HI+ pancreata (785 and 916, respectively, $p = 0.169$). This suggests that the higher endocrine cell mass in HI+ pancreata is the result of islet expansion, (probably through vasodilatation, edema or both) rather than to an increase in the number of islets.

Islet isolation yield in relation to HIs

In a series of isolations in 48 purebred adult and 25 crossbred porcine donors we assessed whether the presence of HIs might influence isolation outcome. Lower yields were found in HI+ pancreata compared to HI- pancreata in both purebred and crossbred pigs, but these differences were not significant ($p = 0.125$ and $p = 0.190$, respectively) (Fig. 3A,C). However, these outcomes may be confounded by the higher endocrine cell mass in HI+ pancreata given that pancreata with higher endocrine cell mass produce higher yields simply because there is more endocrine cell mass to begin with. Adjusted for the differences in endocrine cell mass, significantly lower yields were found in HI+ pancreata compared to HI- pancreata in both purebred and crossbred pigs ($p = 0.03$ in both groups) (Fig. 3B,D).

To assess the influence of the severity of the hyperemia on the yield/endocrine cell mass ratio, the HI+ pancreata were subdivided into HI+ and HI++ pancreata. One-way analysis of variance showed that the mean yield/endocrine cell mass ratio differed between the groups in both purebred and crossbred pigs ($p = 0.007$ and $p = 0.02$, respectively). Bonferroni's test revealed that the yield/endocrine cell mass ratio was significantly higher in the HI++ group compared to the HI- group ($p =$

0.02) in purebred pigs. In crossbred pigs the yield/endocrine cell mass ratio was also significantly higher in the HI++ group compared to the HI- group (p = 0.02). No significant differences were found between HI- vs HI+ and HI+ vs HI++ pancreata in both groups.

Linear regression analysis showed that the yield/endocrine cell mass ratio after was significantly lower in pancreata for every increase in the percentage of HIs in both purebred and crossbred pigs (B = -115.90, p = 0.005 and B = -106.25, p = 0.05, respectively).

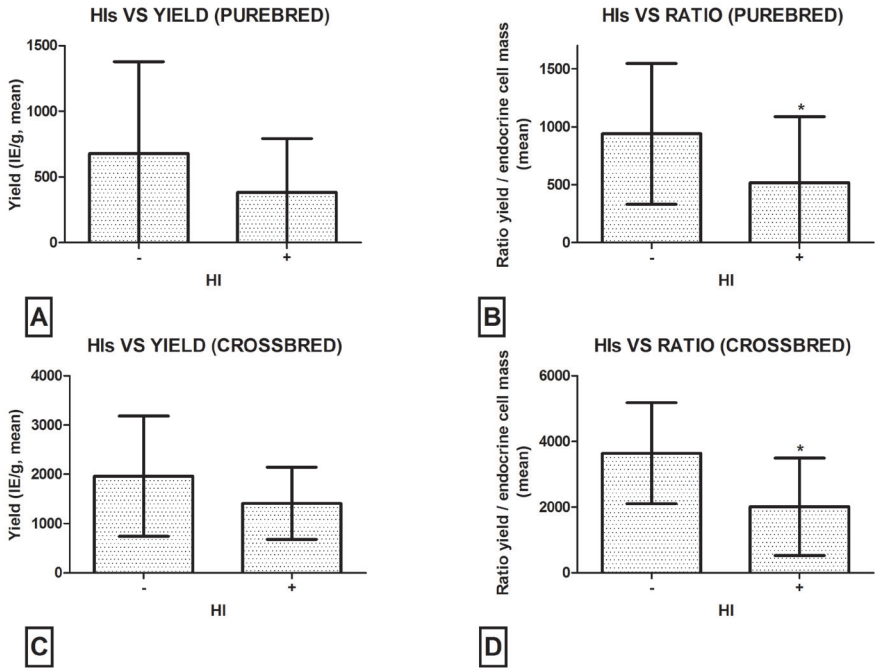

Figure 3: Islet isolation yield and yield/endocrine cell mass ratio in relation to hyperemic islets.
The bars represent the mean islet isolation yield (A,C) and the mean yield/endocrine cell mass ratio (B,D). The error bars represent the standard deviation.
(A,B) Pancreata from purebred pigs (A) Mean yield in HI- pancreata was 679.2 ± 697.5 IE/g. In HI+ pancreata this was 383.4 ± 408.9 IE/g. (B) Mean yield/endocrine cell mass ratio was significantly lower in HI+ pancreata *p = 0.03
(C,D) Pancreata from crossbred pigs. (C) Mean yield in HI- pancreata was 1960.5 ± 1220.6 IE/g. In HI+ pancreata this was 1411.0 ± 733.6 IE/g. (D) Mean yield/endocrine cell mass ratio was significantly lower in HI+ pancreata *p = 0.03

DISCUSSION

Similar to our findings in human donor-pancreata (12), a remarkably high number of hyperemic islets was encountered when studying histological characteristics of the porcine pancreas in relation to islet isolation. Similar islets have been reported

anecdotally in the literature but no mechanisms were described regarding their origination (7-11). Also, no relevance has been determined from the perspective of the isolation of islets for transplantation. Although a slight hyperemia in islets is seen in normal metabolic situations when higher insulin release is demanded (11,15), the phenomena encountered in the present study are far more dramatic and are most likely not related to the normal glucose metabolism. In the literature only one study mentioned the finding of dilated blood vessels in islets, caused by congestion (7). The authors excluded the affected porcine pancreata from their study and gave no data on incidence and severity of the phenomenon, or on possible consequences for isolation outcome.

In the present study, HIs were found in 48% of the pancreata in purebred and in 68% of crossbred pigs. However, the HI+ category consisted of pancreata with at least 1 HI. This could potentially lead to an overestimation of the HI- group, when a hyperemic islet was missed by an observer or when an islet appeared just outside the sectioning plane. However, because 2 observers independently assessed the tissue samples with good agreement and since we have found no differences in the presence of HIs between different parts of the pancreata, it is unlikely that our results were influenced by this. Even if such a misclassification would have occurred, it would have lead to an underestimation of the relationship between HI and isolation outcome, given that HI+ pancreata have lower yields than true HI- pancreata. As a result, the data presented here are a conservative estimate of the strength of the relationship between HI and isolation outcome. Furthermore, we have found similar results in our study of 102 human donor-pancreata (12). In this previous study, HI's were found in 54.6% of all human donor-pancreata, with 4.5% of all islets in the affected pancreata being hyperemic. The affected human pancreata contained slightly more endocrine tissue, and produced significantly lower yields similar to the findings in porcine pancreata. It thus seems to be a general phenomenon occurring in about half of the pancreata.

We have investigated the possible relevance of the presence of HIs from the perspective of islet isolation. A higher endocrine cell mass was found in the affected pancreata. Although the increased endocrine volume was paralleled by an increase in islet numbers, a straightforward comparison is confounded by the fact that only islets of greater than 50 μm were counted. To elucidate this, we have assessed the average beta cell/islet area ratio per islet and furthermore counted all islets, including islets < 50 μm, in a limited series of 22 HI+ and 22 HI- pancreata. We found a significantly lower beta cell/ islet area ratio in HI+ pancreata, without a difference in the total number of islets compared with HI- pancreata. This suggests that islets in these pancreata are more swollen, probably through vasodilatation, edema or both.

When we corrected for the fact that the HI+ pancreata contained significantly more endocrine tissue, significantly lower yields in the HI+ pancreata were seen compared to the HI- pancreata in both purebred and crossbred pigs. This is remarkable

since in HI+ pancreata only a small percentage of all islets was histological abnormal. Most likely, the substantial increase in islet volume, or the reduced isolation outcome is not caused by this small fraction itself. A possible explanation could be that the abnormal islets are just an indicator of the actual damage of the islets. No macrophages or iron deposits were seen and no evidence for an ongoing chronic process was found, a possible explanation is that the HIs arose shortly before the exsanguination and death of the animal and that for instance a sudden rise in blood pressure is responsible for the formation of HIs. In veterinarian literature, it is widely recognized that the slaughtering process induces a rise of the blood pressure with hemorrhages of different extent (16-18). However, further research is needed into the mechanisms by which these hyperemic islets arise e.g. by comparing pigs killed by electric stunning to pigs killed by another method. In our human study, prolonged ICU stay and pre-procurement hypertension were associated with the presence of HIs (12). On the other hand, HIs were also found in 43.3% of "normal" pancreatic tissue samples obtained from elective surgical procedures. So apparently the presence of HIs is not limited to donor organs. However, these "elective patients" were most likely not admitted to the ICU before undergoing surgery and were probably hemodynamically more stable than the pancreas donors. Therefore, it seems likely that, besides the pre-procurement hemodynamic status, the handling of the pancreas during surgery could also be a contributing factor in the development of HIs. This could also be a contributing factor in porcine pancreata.

We have found a relation between the severity of the hyperemic islets in the pancreata in relation to the endocrine cell mass and number of islets, with an increase in severity of the hyperemia being paralleled by a higher endocrine cell mass and number of islets. On the other hand, an increase in severity of the hyperemia did not seem to have a relation with isolation outcome. However, the absence of such a statistical difference could have been the result of the numbers in our study being too small, since there is a statistically lower isolation outcome in HI+ pancreata when compared to HI- pancreata. Furthermore, linear regression analysis showed significantly lower isolation outcome in pancreata containing a higher percentage of HIs. We have found similar results in human donor-pancreata (12). To establish the importance of HIs for islet transplantation, further research, involving post-purification yield, purity, viability etc. would be necessary.

Taken together, we hypothesize that hyperemic islets in porcine pancreata are just an indicator of the actual damage of the islets in the pancreas. These damaged islets could be more prone to fragmentation during the isolation procedure, and thus have more chance to become smaller than 50 μm, thereby resulting in lower isolation yields given the common practice of not including islets smaller than 50 μm when determining the isolation yield. This hypothesis should be tested in future research.

In conclusion, these data confirm that the presence of hyperemic islets may negatively influence isolation outcome in porcine pancreata as they were shown to do in human pancreata and possibly affect the islet quality in the procurement procedure. It is tempting to speculate that this eventually may explain part of the generally experienced difficulties in obtaining sufficient amounts of porcine islets.

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