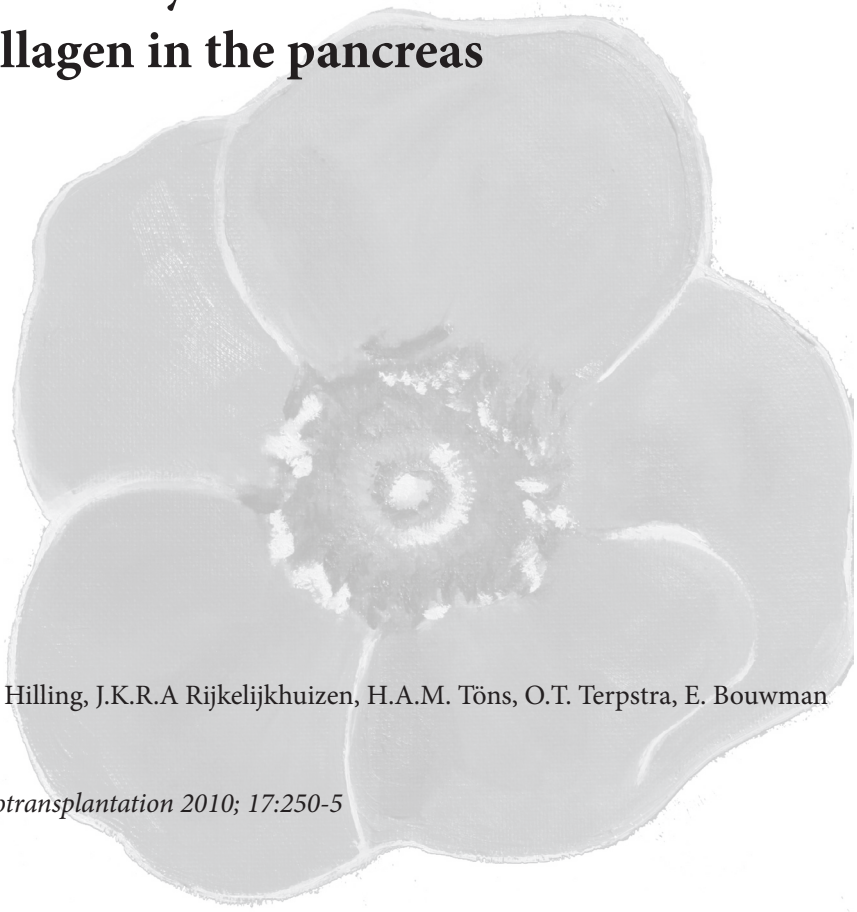
Chapter 7

Porcine islet isolation outcome is not affected by the amount and distribution of collagen in the pancreas



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Xenotransplantation 2010; 17:250-5

ABSTRACT

Background

Variable islet yields in porcine islet isolation may be caused by the collagen substrate within the pancreas. The aim of the present study was to determine the total amount and distribution of collagen within porcine pancreata and their relationship to islet isolation outcome.

Method and Materials

A total of 64 juvenile and 76 adult porcine pancreata of eight purebred breeds were histologically examined. The amount of collagen was quantitatively assessed in tissue samples stained with Sirius Red. Collagen distribution was semi-quantitatively determined by assessing the presence of collagen in the endocrine-exocrine interface and within the islet, in tissue samples stained with Sirius Red and anti-insulin. Islet isolation was performed in 58 pancreata of the adult group.

Results

Total collagen content and islet encapsulation ranged widely in both adult and juvenile pigs. However, the majority of islets in adult and juvenile pigs had no or only a limited collagen capsule. The difference in collagen content between adult and juvenile pigs could not be explained by age. Furthermore, no differences between adult and juvenile pigs were found in islet encapsulation or the amount of intra-islet collagen. In adult pigs, no significant relationships were found between obtained islet yield and total collagen content, islet encapsulation or amount of collagen within the islet.

Conclusions

Considering the limitations in experimental design (staining method) and study material, isolation outcome does not seem to be affected by the total collagen content or collagen distribution. The influence of other matrix elements and collagen subtypes should be investigated.

INTRODUCTION

The pig is considered to be a potentially alternative for human donors of islets of Langerhans (1-5). However, porcine islet isolation procedures have been shown to be notoriously difficult. A possible explanation could be related to donor age and to the relative fragility of the islets of juvenile pigs (4,6,7). Collagenase digestion of the young porcine pancreas usually results in a complete dissociation of both the exocrine and endocrine tissue (7). On the other hand, islet isolation procedures using pancreata from adult pigs have resulted in large islet yields (8-10).

Furthermore, despite improvement of isolation procedures, islet isolation is still associated with a considerable loss of endocrine tissue. This indicates that collagenase digestion of the pancreas is not limited to the exocrine pancreas but affects the islets as well. Because collagen is the major target in the enzymatic dissociation of the pancreas, the collagen substrate within the pancreas is one of the variables that could account for the unpredictable, highly variable islet yields (7,10-12). As successful islet isolation depends upon effective separation of islets from exocrine tissue, a more detailed knowledge of the composition of the connective tissue of the pancreas on which collagenase is acting is necessary.

Previous studies reporting on the collagen content of porcine pancreata have based their conclusions on a small number of animals and furthermore, did not correlate their results to the actual isolation outcome of the pancreata used in their collagen assessment (10-14).

The aim of the present study was to determine the total amount and distribution of collagen within a large study population of adult and juvenile porcine pancreata and assess the relationship of these determinants to the outcome of islet isolation in adult pigs.

MATERIALS AND METHODS

Organ Procurement and Pancreatic Sampling

A total of 140 purebred porcine pancreata were harvested in different slaughterhouses. The population consisted of a juvenile group (6 to 12 months) of 64 animals and involved five different breeds (Great Yorkshire n = 21, Dutch Landrace n = 25, Norwegian Landrace n = 2, Large White n = 9, Finnish Landrace n = 7). The adult group (12 to 78 months) consisted of 76 animals and involved eight different breeds (Piétrain n = 8, Great Yorkshire n = 10, Dutch Landrace n = 15, Norwegian Landrace n = 7, Large White n = 7, Hampshire n = 5, Finnish Landrace n = 14, Duroc n = 10). All animals were killed by electric stunning and exsanguination. Warm ischemia times were between 20 and 30 minutes. After dissection on ice, 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 and 5 h. Biopsies were taken, immersed in Bouin's fixative, cleared and stored in ethanol 70%.

Amount of collagen

To detect collagen, paraffin-embedded tissue samples of 4μm were stained with 0.1% Sirius Red F3B (Klinipath, Duiven, The Netherlands) in a saturated solution of picric acid for 90 minutes, washed with a saturated solution of picric acid, dehydrated and cleared with xylenes. Sirius Red, when dissolved in a saturated solution of picric acid, stains both structural as well as basement membrane collagen and consequently stains all types of collagen without differentiating between collagen subtypes (15-17).
Quantitative measurements were made using the Zeiss KS-400 image analysis system (Carl Zeiss Ltd, Welwyn Garden City, Herts, UK). The collagen stained area was quantified by the assessment of 30 random fields in each tissue sample (Fig. 1 A, B). The KS-400 image analysis system quantified the red stained area per field, by calculating the percentage of red staining in each field. The average percentage of red staining in these 30 fields was considered to be a valid representation of the amount (percentage) of collagen in the whole pancreas.

Distribution of Collagen

Distribution of collagen was evaluated in 4-μm sections of the same paraffin-embedded tissue samples as used in the determination of the amount of collagen. Collagen was stained with 0.1% Sirius Red F3B (Klinipath, Duiven, The Netherlands) and the identification of islets was facilitated by staining the beta cells with a polyclonal guinea-pig anti-porcine insulin antibody (Zymed, Invitrogen, Carlsbad, USA) at a dilution of 1:200 overnight. To visualize the immunoreactions, sections were incubated with 3,3'-diaminobencidine (DAB) dissolved in Tris/HCl (0.05 M) with 15 μl H_2O_2.

Distribution of collagen was determined by assessing the presence of collagen within the islet and in the endocrine-exocrine interface (the "islet-capsule"), in an average of 100 islets per tissue sample. The presence of collagen in the islet-capsule (prior to isolation) of each assessed islet was scored in four categories: 0% to 25%, 25% to 50%, 50% to 75%, 75% to 100% collagen encapsulation (Fig. 1 C,D). The average score of all islets in a tissue sample was taken to represent the islet encapsulation with collagen in the whole pancreas. The amount of collagen within the islets was scored semi-quantitatively: 0 (absent), 1 (intra-islet collagen present in $\leq$ 25% of islet area), 2 (intra-islet collagen present in >25% of islet area) (Fig. 1 E,F). The average score of all islets in a tissue sample was considered to be a valid representation of the amount of collagen within the islets in the whole pancreas.

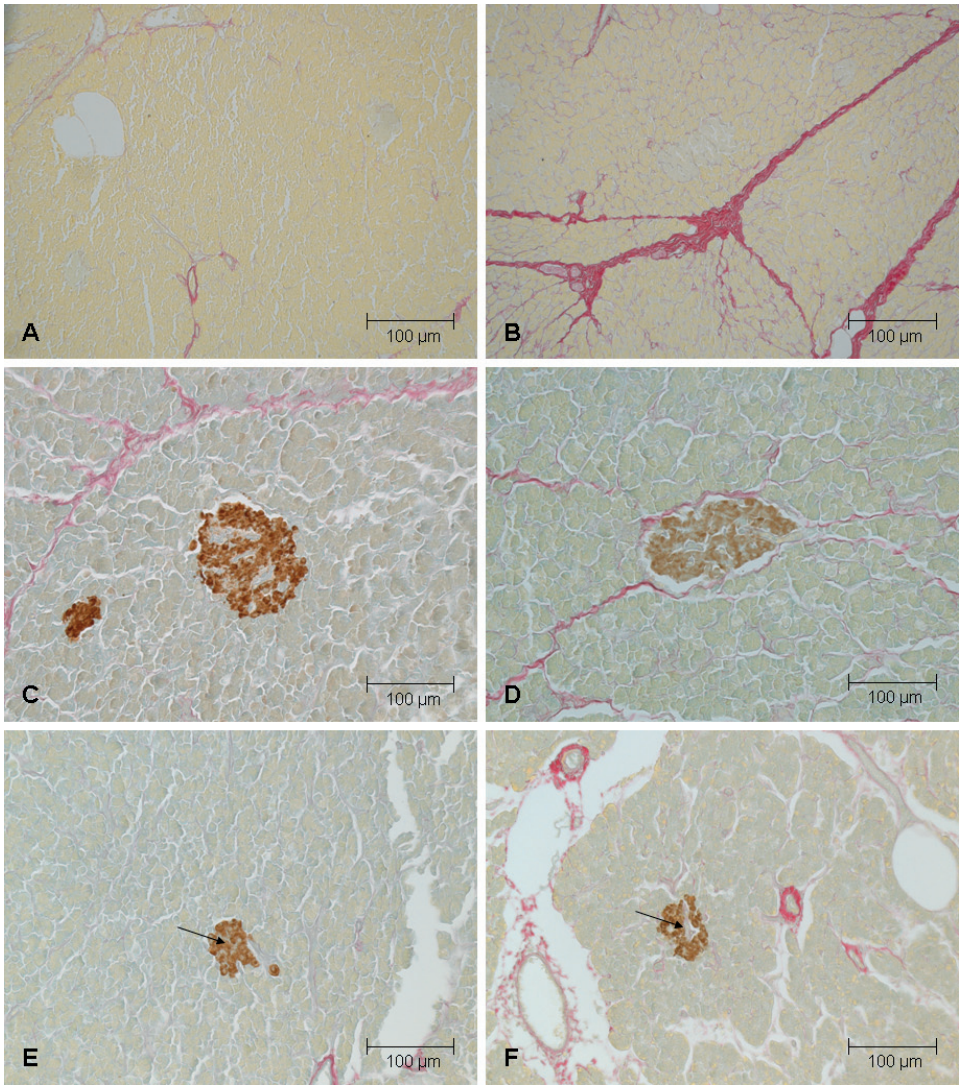

Figure 1: Total amount of collagen and collagen distribution
A,B) The total amount of collagen (red) was determined in tissue samples stained with Sirius Red in a saturated solution of picric acid. The collagen stained area was quantified by the assessment of 30 random fields in each tissue sample
A) 1 of the 30 fields of a pancreas with an average amount of collagen of 3.0%. This field displays part of the exocrine tissue of the pancreas neck B) 1 of the 30 fields of a pancreas with an average amount of collagen of 10.2%. This field displays part of the exocrine tissue of the pancreas neck.
C,D) Collagen encapsulation (red) was assessed in tissue samples double stained with Sirius Red and anti-insulin (brown)
C) Islet with 0-25% collagen encapsulation D) Islet with 75-100% collagen encapsulation
E,F) Intra-islet collagen (arrows) was assessed in tissue samples double stained with Sirius Red and anti-insulin (brown)
E) Islet with no visible intra-islet collagen, score: 0 F) Islet with intra-islet collagen present in <25% of the islet area, score: 1

Islet isolation

Islet isolation was performed in 58 porcine pancreata of the adult group. The isolations in this study were performed after obtaining sufficient experience with porcine islet isolations to achieve consistent results. The pancreata were cut clean in the laboratory. The arm of the pancreas (20 to 40 g in weight) was intraductally injected with a solution of Liberase PI (0.5 mg/ml, Roche, Basel, Switzerland) in University of Wisconsin solution. The distended tissue was cut into pieces and incubated in HBSS at 37°C for 20 to 30 min. The digested tissue was placed in a beaker on ice, which was manually shaken to dissociate gently and dilute the tissue. Subsequently, the tissue was poured over a filter with 1000-μm mesh and washed in cold HBSS for three 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.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 statistical software (SPSS Inc., Chicago, IL, USA). After visual inspection of the data, these were found to be normally distributed so that parametric tests were used. Juvenile and adult pigs were compared by the parametric student *t*-test. To determine the effect of age on collagen content and islet encapsulation, multiple linear regression was performed with either collagen content or islet encapsulation as dependent factors and groups of pigs (juvenile vs. adult) and age in months as independent variables. One way analysis of variance was used to compare isolation outcomes between different breeds. Multiple linear regression was performed with yield as dependent factor. Independent factors were either total amount of collagen, islet encapsulation or intra-islet yield, breed and age in months. P values ≤ 0.05 were considered to be statistically significant.

RESULTS

Amount of Collagen

The mean collagen content (± SD) in the adult group was 5.5 ± 2.0%, this was significantly higher ($p < 0.001$) than in the juvenile group where this was 3.7 ± 1.3% (Fig. 2A). The collagen content ranged in adult pigs from 1.7 to 11.0% and in juvenile pigs from 1.1 to 7.1%, showing a widespread range in collagen content between pancreata.

When we corrected for age in months, a difference in collagen content in juvenile vs. adult groups still remained ($B = -2.08$, $p < 0.001$). However, this difference

could not be explained by the age in months (B = -0.01, p = 0.40), and must therefore be explained by another (unknown) difference between these groups.

In a limited series of 15 (randomly selected) pancreata, the collagen content was assessed in tissue samples from two different parts of the pancreas. No clear differences in collagen content were observed between the different parts of the pancreas (results not shown).

Collagen distribution

In adult pancreata, the mean degree of encapsulation of the islet with collagen was 38.9 ± 12.8%. In juvenile pancreata, the mean percentage was 35.2 ± 8.7% (p = 0.04) (Fig. 2B). This percentage ranged in adult pigs from 14 to 78% and in juvenile pigs from 19 to 64%, showing a widespread range in islet encapsulation between pancreata.

However, when adjusted for age in months, no difference in islet encapsulation between adult and juvenile groups was found (B = -4.76, p = 0.09).

The majority of the assessed islets was 0% to 25% encapsulated with collagen. Moreover, 82% of the assessed islets in adult and 88% in juvenile pigs was encapsulated < 50% (Table 1).

We found no significant difference between juvenile and adult pigs with regard to the presence of collagen within the islet (p = 0.69) (Fig. 2C). In all instances, collagen was exclusively located around the capillaries.

In a limited series of 15 (randomly selected) pancreata, the islet encapsulation and intra-islet collagen were assessed in tissue samples from two different parts of the pancreas. No clear differences in islet encapsulation or intra-islet collagen were observed between the different parts of the pancreas (results not shown).

Amount of collagen and collagen distribution vs. islet yield

After isolation, the average obtained islet yield in 58 adult pigs was 545.4 ± 601.2 IE/g pancreas (range 18 to 3849). One way analysis of variance showed that the mean obtained yield did not differ between the different breeds (p = 0.28)

When corrected for breed and age in months, no significant relations were found between isolation outcome and collagen content, islet encapsulation and intra-islet collagen (B = -33.28 p = 0.52, B = -1.51 p = 0.81 and B = 103.02 p = 0.85, respectively).

Table 1. Percentage collagen encapsulation in adult and juvenile pancreata

	Percentage collagen encapsulation, mean ± SD			
	0-25%	**25-50%**	**50-75%**	**75-100%**
Adult pigs (n = 76)	59.6 ± 21.3	22.6 ± 8.8	10.6 ± 8.9	7.3 ± 7.7
Juvenile pigs (n = 64)	68.7 ± 16.8	19.7 ± 8.8	7.1 ± 6.2	4.6 ± 4.2

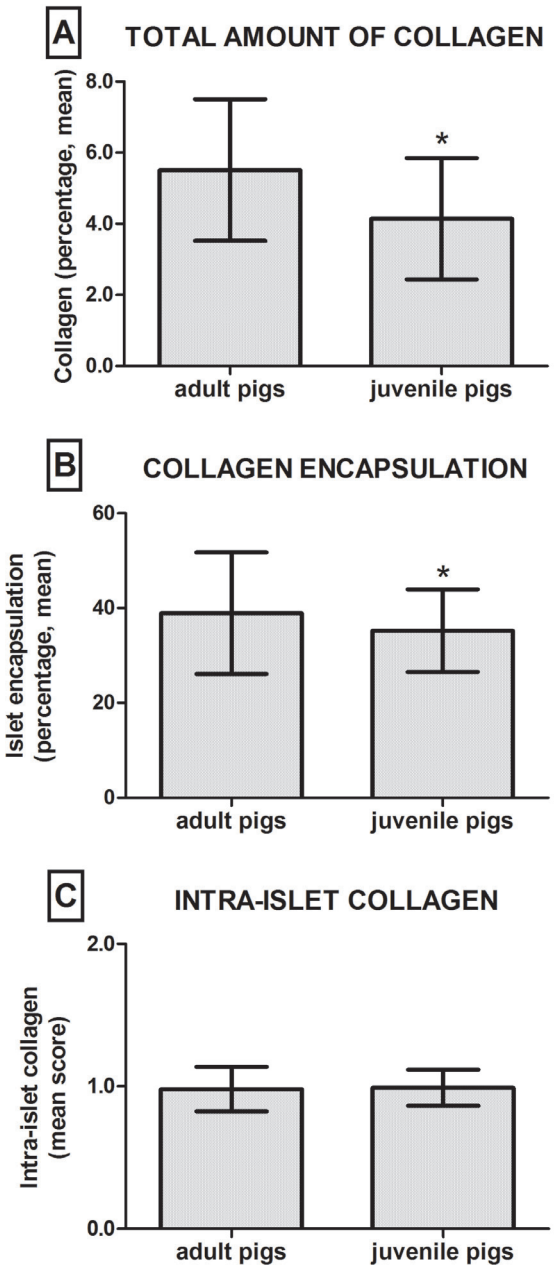

Figure 2: Total amount of collagen and collagen distribution

The bars represent A) the mean percentage of collagen B) the mean percentage of the circumference of the islets that is encapsulated with collagen C) the mean score for intra-islet collagen. The error bars represent the standard deviation.

A) The mean percentage of collagen in adult pigs was significantly higher than in juvenile pigs, * $p<0.001$

B) The mean islet encapsulation with collagen was significantly higher in adult than in juvenile pigs * $p=0.04$

C) No significant differences ($p=0.94$) in intra-islet collagen were found between adult and juvenile pigs

DISCUSSION

Variable yields in porcine islet isolation may be caused by the collagen substrate within the pancreas. If this were true, we would be able to select eligible pigs in advance, to achieve higher isolation yields. However, considering the limitations in experimental design (staining method) and study material, in our study of 58 adult pigs we failed to observe a relationship between isolation outcome and total amount of collagen, islet encapsulation and intra-islet collagen. Juvenile pigs are generally shown to produce lower islet yields than adult pigs and collagenase digestion usually results in complete dissociation of the pancreas (7-10). These contrasting results may also be caused by differences in the collagen substrate between adult and juvenile pigs. However, because we found no relationship in adult pigs and the variance of total amount of collagen, islet encapsulation and intra-islet collagen was even smaller in juvenile pigs, it is unlikely that these would have an effect on isolation outcome in juvenile pigs.

The total amount of collagen in porcine pancreata has been considered to be an important factor in determining the isolation result (11). Yet our results do not support this., the difference in collagen between adult and juvenile pigs in our study could not be explained by the age-difference, but may be explained by another (unknown) difference between those groups. A collagen capsule surrounding the islet could potentially provide protection against enzymatic disintegration of islets and consequently their fragmentation, it has been suggested that a factor in the differing results is a more extensive capsule surrounding the islets of the adult pig pancreas as compared to the young pig pancreas (7,10). However, in our study, we found no difference in islet encapsulation between adult and juvenile pigs. Both adult and juvenile pancreata had no or only a very limited collagen capsule. This is in accordance with van Suylichem et al. (11) and van Deijnen et al. (13) who found that in pigs, the adhesion between islets and exocrine tissue almost exclusively depends on cell-to-cell adhesion.

Our isolation results could have been influenced by (i) the isolation method and (ii) the procurement method and pancreas sampling. The isolation method we used is an accepted method of islet isolation, which is performed in many centers in every day practice and provides consistent results. Even though other methods may result in higher yields, it is unlikely that the observed relationship between isolation outcome and collagen was influenced by the isolation method. Ideally, pancreas samples should be taken before any manipulation takes place and warm ischemia times should be as short as possible. However, as the same procedure was used for all the pancreata in our study, it is unlikely that it will explain any relationship between collagen and isolation outcome.

In the present study, Sirius Red was used to stain collagen. Hence, our observations concern the amount and distribution of collagen in general, without differentiating between subtypes. Previous studies have shown that collagen types I,

III, IV and VI are present in the peri-islet capsule of the porcine pancreas (12,18). Compared with rat, human, and canine tissues, expression of most collagen types and laminin in the porcine pancreas seems to be rather weak (11). It can be speculated that the composition of the islet capsule, and the relative concentration of the components, could influence islet isolation outcome and possibly confound the observed relation between islet isolation outcome and the complete islet capsule. However, as we found no or only a very limited collagen capsule in our study population when we stained tissue samples for all types of collagen, we expect that collagen subtypes play no or only a minor role.

In conclusion, although collagen is the major target in the enzymatic dissociation of porcine pancreata and although total collagen content and collagen distribution show high variability, we found no relationship between islet isolation outcome and these determinants in adult pigs. However, other matrix elements and collagen subtypes could play a role and should be further investigated.

ACKNOWLEDGEMENTS

The authors would like to thank Dr. P.J. Marang-van de Mheen for the statistical advice and K.G. van der Ham for the images of the total amount of collagen, islet encapsulation and intra-islet collagen.

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