

PANCREATIC ISLETS: ISOLATION AND PURIFICATION METHODS

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METHODS FOR ISOLATION AND PURIFICATION OF JUVENILE AND ADULT PIG ISLETS

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ABSTRACT

The current situation of organ transplantation is mainly determined by the disbalance between the number of available organs and the number of patients on the waiting list. This obvious dilemma might be solved by the transplantation of porcine organs into human patients. The metabolic similarities which exist between both species made pancreatic islets of Langerhans to that donor tissue which will be most likely transplanted in human recipients. Nevertheless, the successful isolation of significant yields of viable porcine islets is extremely difficult and requires extensive experiences in the field. This review is focussing on the technical challenges, pitfalls and particularities that are associated with the isolation of islets from juvenile and adult pigs considering donor variables that can affect porcine islet isolation outcome.

INTRODUCTION

While the early era of modern organ transplantation was challenged by the rapid rejection of the transplanted tissue [1], one of the main problems of organ transplantation in the current year is the imbalance between the number of available donor organs and the number of patients on the waiting list. Recent data from the North American United Network for Organ Sharing (UNOS) show that the donation rate increased by 94% over a period of more than twenty years. This significant increase has been nullified by the simultaneous expansion of the waiting list by 253%. The worldwide equivalent figure, as provided by the World Health Organization, is an organ supply-to-demand ratio of 1:25 [2]. This obvious dilemma could potentially be solved by the transplantation of xenogeneic organs into human patients. Due to the anatomical and physiological similarities between humans and pigs, porcine donors are the species of choice to fill the gap between the demand and supply of human organs. However, the metabolic and endocrine concordance between both species made pancreatic islets of Langerhans to that donor tissue which will be most likely transplanted in human recipients in clinical trials [2-4]. In fact, the first series of clinical islet xenotransplantation was performed in 1993 and involved transplanting fetal pig islets into ten insulin-dependent patients with type 1 diabetes. Although small amounts of porcine C-peptide could be detected in the urine for more than one year, a reduction of exogenous insulin demand was not achieved in any of the recipients [5]. Subsequent approaches provided much more promising results demonstrating viable tissue nearly ten years after transplantation of encapsulated neonatal pig islets into a non-immunosuppressed patient with type 1 diabetes [6]. The finding that the vigorous antibody- and complement-mediated rejection that is observed after pig-to-primate xenotransplantation [1, 7] can be reduced using adequate immunosuppression [8, 9], anti-clotting treatment [10] or multi-transgenic donor pigs [11-13] makes clinical trials for pig islet xenotransplantation a realistic option for providing a widespread treatment for a larger population of patients with type 1 diabetes [14].

However, before treatment of type 1 diabetic patients with pig islets becomes a daily reality, numerous precautions must be taken to establish islet xenotransplantation as a safe procedure. Specific pathogen-free (SPF) breeding in biosecure facilities is a mandatory measure to prevent transmission of zoonotic pathogens to the human population [15]. The costs of running these highly specialised facilities will have an impact on the total costs for each islet isolation that are likely to exceed the costs for human islet

allotransplantation [16], particularly if pancreases from adult pigs older than 2 years are processed.

The immunological, technical and economical advantages and disadvantages of using pancreases retrieved from fetal, neonatal, juvenile or adult pigs have been extensively discussed [17-19]. This review will rather focus on the technical challenges, pitfalls and specifics that are associated with the isolation of islets from the pancreas of pigs of approximately 6 months to older than two years.

PIG DONOR VARIABLES

Pig strain:

Islet isolation always starts with donor selection. The selection of pig strains suitable for successful release of intact islets from the pancreas is of relevance for islet research but is of outstanding importance for clinical xenotransplantation considering the immense costs related to breeding and maintenance of SPF pigs in biosecure facilities.

The very few histomorphologic studies in native pig pancreases which have been performed so far clearly revealed that significant differences in terms of islet size, shape and number exist between different pig strains such as German Landrace, Duroc, Piétrain or wild boars. In two studies it was found that pancreases of wild boars have a high density of islets of very small diameter while the German Landrace is characterized by a high percentage of large islets resulting in low and high total islet volume, respectively [20, 21].

Duroc and Piétrain pigs seem to have an islet volume in between these extreme poles.

However, to categorize the suitability of different strains for successful islet isolation the expression of the peri-islet connective tissue matrix has to be considered as well. Two studies reported in agreement that Duroc pigs have a low expression of the peri-islet capsule while the German Landrace and the German Large White Pig (Deutsches Edelschwein) are characterized by a strong expression of this structure [20, 22] that seems to protect islets from the harmful effects of proteolytic enzymes used for pancreas digestion. Despite the differences that exist in the thickness and perimeter of the peri-islet capsule it is remarkable how similar the capsular composition of different extracellular matrix (ECM) proteins is in widely disparate strains such as Piétrain, Goettinger Minipig or wild boar [22].

We are aware of only a few studies that have correlated the strain of the donor pigs with porcine islet isolation outcome. In agreement with the histomorphologic studies of Kirchhof et al. and Meyer et al. [22, 20] we found a clear advantage of isolating islets from the

pancreas of adult Large White Pigs (Deutsches Edelschwein) compared to German Landrace and hybrid pigs [23]. From two other reports we can conclude that Piétrain pigs provided higher islet yields compared to German Landrace and hybrid pigs [21, 24].

There is no doubt that the selection of the pig strain has a significant effect on islet isolation outcome. However, to rate the relevance of these previous observations for the current situation it is important to realize that pig breeding is a continuously ongoing process performed to meet the demands of the consumers [25]. For French Large White Pigs it was demonstrated for the period between 1977 and 1998 that the lean muscle mass increased by 15% while the relative liver weight decreased by 16% [26]. Since no data are available for the pancreas weight we can only speculate that the changes in relative fat and muscle mass may also affect the pancreas as well as islet cell growth and proliferation. However, it has been clearly shown in different livestock species that a high lean muscle mass inversely correlates with the fat content of the carcass which is mainly under the control of the insulin-glucose metabolism [27]. In agreement, pigs with a lean phenotype have a relatively higher somatotropin serum level compared to pigs with an obese phenotype which are characterized by a relatively higher insulin level or a larger insulin response toward stimuli [28-31]. Regardless of ease of islet separation, it can be anticipated that the islet volume within the pancreas of pigs is continuously reduced as long as pig breeding is mainly aiming on a higher percentage of lean muscle mass in the carcass.

Within a short period of approximately 3 years (2006 – 2008) we observed a significant increase of the variability in isolation outcome when islets were isolated from the pancreas of BHZP (German Federal Hybrid Breeding Programme) pigs. As shown in Figure 1, this observation correlated with an alteration in the morphology of islets assessed in the pancreas of donor pigs of the identical strain. While samples retrieved before 2006 showed native islets with an ovoid shape well demarcated from acinar tissue (Figure 1A), islets assessed in the same strain after 2008 were characterized by a smaller diameter or a dumbbell-like shape (Figure 1B).

Figure 1. Insulin immunostaining of native pancreas sections retrieved from BHZP (German Federal Hybrid Breeding Programme) retired breeder pigs before 2006 (A) and after 2008 (B) (magnification x 10).

However, when considering the enormous costs for housing, breeding and maintenance of adult SPF strains [15, 16], miniature pigs will become more and more important as

potential donors for clinical islet xenotransplantation. Future efforts should therefore be aimed to crossbreed obese farm pig strains identified as effective islet donors with suitable strains of miniature pigs [32].

Porcine donor age:

One important variable to be discussed in the context of donor selection is the donor age. First attempts to isolate islets from the pancreas of market age pigs revealed an unique fragility of islets which appears to be more pronounced than in any other species investigated so far. During the isolation procedure juvenile islets fragmented into small clusters or single cells [33]. This may be explained by the weak expression of ECM proteins in pig pancreases, particularly in the peri-islet region, when compared to other species such as rat, dog and human [34, 35]. The fragmentation of pig islets may be accelerated due to the particular specific cytoarchitecture of pig islets composed of several smaller subunits with a beta-cell core and an alpha-cell mantle [36]. Further studies have indicated that islets isolated from adult pigs are morphologically more stable and are easier isolated compared with juvenile pig islets [32, 37, 38].

Although individual differences exist in young and old pigs [39, 40] pancreases from retired breeders contain a higher frequency of larger and compact islets compared with juvenile pig pancreases which are defined by a significantly higher density of islets partially appearing as small clusters composed of only a few cells [21, 41-43]. The aging process does not only contribute to increased islet size but seems also to enhance the total collagen content in pig pancreases which may reflect the age-dependent formation of a protective matrix of ECM proteins around islets [44]. Indeed, we (unpublished data) and others observed the presence of a peri-islet capsule in adult pig pancreases but not in young pigs [38]. In agreement, in a small number of samples it was found that expression level of laminin, fibronectin, collagen type I, III and IV is higher in old pigs compared to young pigs [22]. In contrast, another report revealed that the pancreas of juvenile pigs contains a higher total collagen amount than adult pigs [45]. Subsequent studies could not confirm a difference between young and old pigs regarding the formation of a peri-islet capsule or the histologic distribution of different collagen types. Among all collagen types assessed, collagen VI was the most abundant intra- and peri-islet collagen type [46]. This is particularly remarkable as collagen VI seems to be resistant towards cleavage by bacterial crude collagenase [47]. However, another study could not detect any collagen VI in or around islets collected from different strains [22].

Nevertheless, it seems to be important to note that the age of the donor pig does not only determine the separability of islets but also the *in vitro* as well as *in vivo* function of the islets. It was shown that a higher islet volume of juvenile pig islets is needed to restore normoglycemia in diabetic rodents when compared with posttransplant function of adult pig islets [37, 41, 42, 48]. This, again, seems to reflect the morphological disintegration of juvenile pig islets at the periphery caused by the isolation process and demonstrated by the loss of peripheral alpha cells [49, 50].

Overall, the majority of studies clearly demonstrate that adult pig pancreases are characterized by a larger number of compact islets and a larger functional islet capacity compared to juvenile donors, but the data about the expression of ECM proteins are very inconsistent. The contradiction between different reports may reflect the use of heterogeneous tissue samples and the application of non-specific ineffective antibodies.

PIG PANCREAS PROCUREMENT

The method of choice for pig pancreas procurement depends on the scientific purpose and on the financial resources available. Generally, two different strategies are available to procure a pig pancreas. While the slaughterhouse procedure is straightforward, it carries the risk of extensive bacterial contaminations and exposure to significant warm ischaemia. However, a surgical approach is complex and associated with enormous costs. The logistical requirements of a pig pancreas procurement procedure according to clinical standards seem to favour the selection of miniature pigs as potential donors for islet xenotransplantation in patients with type 1 diabetes.

Nevertheless, apart from financial and logistical considerations the few reports published so far indicate the detrimental effects of a surgical pancreas procurement on pig islet isolation outcome [51, 52] when compared with a slaughterhouse procedure that involves immediate brain death induction, exsanguination and rapid evisceration to minimize warm ischaemia time [53]. Whether these observations are related to the use of unsuitable perfusion media, excessive perfusion pressure or to physiological stress caused by extensive medication and surgical trauma remains unclear [54].

Regardless which technique is selected for pig pancreas procurement, our own experience, as well as that of others, clearly indicates that the removal of blood from pancreatic tissue is absolutely essential to ensure proper activity of intraductally perfused enzymes. The underdigestion of pancreatic tissue, resulting from the inhibitory effect of residual blood on enzyme activity, can cause a substantial reduction in islet yield [55, 56].

Intraductal pancreas perfusion technique:

Intraductal pancreas perfusion is a backbench procedure that has a duplicate function: first, it decreases rapidly the core temperature of the pancreas in order to reduce detrimental effects of warm ischaemia without the need for intravascular flushing [57]. Second, it is the most efficient way to introduce the enzyme blend into the pancreas.

Manual distension of the pancreas using a syringe is easy to apply as it does not require special equipment. In contrast, the automated pancreas perfusion is performed by means of a complex pump-perfusion device offering the option of continuous pressure control [58, 59]. However, the choice of the distension method depends also on the timing of intraductal pancreas perfusion. Early intraductal pancreas perfusion performed immediately after pancreas retrieval in a slaughterhouse or in a surgical animal facility prior to pancreas shipment to the isolation facility is mostly a logistical challenge that is only be feasible by using a minimum of equipment.

Comparison of manual distension with automated perfusion in human pancreases, has demonstrated the superiority of controlled perfusion over manual syringe loading with regard to islet yield [60]. Although similar comparative experiments have not been performed in the pig pancreas, the findings of Lakey et al. may be particularly relevant for the soft and fragile porcine pancreatic tissue. At 180 mm Hg of perfusion pressure the perfusion medium penetrates islets and expands the intercellular space thereby loosening islet structure and rendering pig islets more sensitive toward disintegration [61]. The penetration of pig islets can only be prevented by using an intraductal perfusion pressure of less than 50 mm Hg [62].

Intraductal pancreas perfusion buffer:

The selection of the most suitable pancreas perfusion medium and/or collagenase solvent is primarily determined by the duration of cold ischaemia time until pig pancreas digestion can be initiated. The current standard medium that is mostly used as enzyme buffer is Hank's balanced salt solution (HBSS). However, the simple composition of HBSS does not allow the preservation of tissue during prolonged cold ischaemia. This is particularly relevant for cold stored pig pancreases which have a lower tolerance for ischemically induced damage than human pancreases [63, 64]. This specific sensitivity for the detrimental effects of ischaemia does not only concern the integrity of acinar tissue [65] but also of the ductal system. The low content of fibrous tissue in the pig pancreas accelerates the collapse of the ductal system during cold storage which prevents efficient

intrapancreatic enzyme distribution at a later time point [66]. Measurements in adult pig pancreases demonstrated that the integrity of the ductal system is also affected by warm ischaemia. It was noted that the intraductal pressure was more than doubled in perfused pig pancreases when exposed to warm ischaemia longer than 20 min [56]. These findings correspond to improved homogenous distribution of infused collagenase and increased islet yield in human pancreases when the ductal system was initially stabilized by intraductal perfusion of Kyoto preservation solution prior to cold storage [67]. Similar observations were made in pig pancreases using University of Wisconsin (UW) solution for intraductal perfusion [57]. These findings clearly suggest that the early intraductal perfusion/distension of the pancreas with UW-solution is particularly relevant when islets are isolated from pig pancreases exposed to significant cold ischaemia [68].

Studies in adult pig pancreases revealed that the replacement of HBSS by UW-solution administered as intraductal enzyme buffer increased islet yield or islet viability even in pancreases processed after short cold ischaemia time [37, 69-71]. In perfect agreement with Heald et al. [70] we found that collagenase concentration has to be increased by 25% to optimize release of cleaved islets when using UW-solution [37] which is in accordance with the observation that UW-solution inhibits collagenase class II activity [72]. Because no data are currently available, we can only speculate whether UW-solution may also affect collagenase class I activity. As a consequence of these considerations different modified or simplified versions of UW-solution were utilized for intraductal pig pancreas collagenase perfusion [71, 73] aiming on omission of potentially inhibitory components such as high potassium, magnesium, adenosine, allopurinol, glutathione and hydroxyethylstarch [74].

The decision whether the collagenase blend has to be administered prior to or after cold storage of pig pancreases seems to depend on the preservation procedure used. While intraductal enzyme perfusion prior to static cold storage improves islet isolation outcome from ischaemic pig pancreases [68, 75], the intraductal administration of enzymes prior to prolonged pig pancreas preservation by means of the two-layer method seems to be detrimental for porcine islet integrity and viability [64].

PIG PANCREAS DIGESTION

The primary goal of pancreas digestion is the release of a maximum number of intact islets from within the surrounding acinar tissue. The requirement to obtain complete dissociation of the non-endocrine components of the pancreas without dispersing the endocrine cell clusters is unique in the field of tissue separation [76] and reflects the dilemma of any

effective islet isolation method to balance two opposite priorities. To achieve this delicate equilibration the vast majority of procedures combine the application of collagenolytic enzymes with mechanical treatment of the gland.

Enzyme blend selection:

Since the structure and consistency of the acinar tissue differs enormously between human and porcine pancreases, enzyme activities need to be adjusted for the digestion of the porcine pancreas which appears to be much more fragile in comparison with the human pancreas. In previous studies we found that the concentration of a crude collagenase mixture needed to efficiently digest a human pancreas has to be reduced by nearly 50% for a retired breeder pig pancreas. Moreover, the enzyme activities were additionally reduced by decreasing the digestion temperature from 37 to 32°C [37]. Nearly identical findings were observed when using purified enzyme blends for adult pig pancreas digestion. While the enzyme concentration was reduced in a range from 50 to 67% compared to the human concentration, the digestion temperature was adjusted between 24 and 28°C [77] which is equivalent to a further reduction of the enzyme activity by 50% [78].

However, the strategy to reduce enzyme activity by decreasing the digestion temperature also has the advantage of reducing the metabolic activity and nutritive demand of islets that are exposed to a detrimental environment. This environment is characterized by anoxia [79], acid pH, hyperosmolarity and the presence of harmful endogenous enzymes [80-82] thereby reducing the morphological and functional integrity of islets. Other attempts have aimed to inhibit trypsin as initial activator of the endogenous zymogen cascade. Although large variability in trypsin activation seems to exist between individual pig pancreases [82], the majority of studies indicate an improvement of islet yield or post purification recovery when the intraductally administered enzyme perfusion medium was supplemented with trypsin inhibitors [83-86].

Previous studies have clearly demonstrated that neutral protease from *Clostridium histolyticum* or *Bacillus thermoproteolyticus* rokko is detrimental for islet integrity [87-89] particularly when used for ischaemic pig pancreases [64]. To reduce the harmful effects of proteolytic enzyme blends, neutral protease was replaced by dispase in the Liberase PI blend (Roche Diagnostics, Indianapolis, USA) specifically developed for porcine islet isolation [90]. Dispace has been shown to be less harmful during dispersion of isolated rat islets when compared with other enzymes [91]. This observation may have implications for the isolation of intact islets as well. As dispase does not cleave laminin (a vital component

of the basement membrane [92, 93]), it can be hypothesized that the loss of the basement membrane, that is usually observed during enzymatic islet isolation [94], is reduced thereby improving islet survival during the isolation procedure, after culture and posttransplant. This enzyme blend has been established as widely used enzyme blend for pig islet isolation [39, 56, 73].

Nevertheless, because evidence is still lacking that dispase has significant advantages compared with neutral protease, our current protocol for adult pig islet isolation utilizes a two-component enzyme blend composed of collagenase and neutral protease (Serva Electrophoresis, Uetersen, Germany) that offers the option to individually reduce the neutral protease activity according to donor variables, pancreas procurement and cold ischaemia time [95, 96].

Dissociation:

In the history of islet isolation, several different techniques have been established and then discontinued as soon as a more advanced method was developed [97]. During this evolutionary process, the automated digestion-filtration method developed by Ricordi still representing the current gold standard for the dissociation of human pancreases for islet release [98]. The automated digestion-filtration device consists of a continuously shaken digestion chamber loaded with the enzyme-distended pancreas together with a number of stainless-steel balls to increase the mechanical dissociation of the tissue. The chamber is topped by a 500 µm-mesh and closed by a conical top. This digestion-filtration device is implemented in a closed circuit during the recirculation phase to allow continuous perfusion of the system with different buffers from the flat bottom of the chamber to its conical top. As soon as fluid samples taken from the digestion circuit demonstrate release of a significant number of cleaved islets the collection phase of digestion is started to recover free islets from enzyme activity. While released islets are collected, the mechanical treatment of the pancreas in the agitating chamber is ongoing as non-digested tissue is retained by the mesh. This system has the great advantage that a final endpoint of the digestion does not need to be determined. This is in contrast to static digestion procedures as practised in the past [99].

Although this technique has been established in all centres for clinical islet transplantation, numerous variations have been introduced over time. These modifications mainly concern the replacement of motor-driven movement by manual agitation of the digestion chamber, the amplitude and frequency of shaking as well as the spatial orientation of top and bottom chamber side during agitation. Our own experiences in porcine islet isolation resulted in a

mixture of automated chamber shaking at the beginning of the recirculation phase and manual agitation at the end of recirculation and during collection phase. During the manual agitation the chamber is frequently turned upside down to facilitate and optimize the release of cleaved islets by means of gravity force which appears to be important because the volume of a distended adult pig pancreas can entirely fill the digestion chamber [77, 95, 96].

However, the variability of techniques for processing pig pancreases is much broader than what has been described for human pancreases and reflects the difficulties successfully releasing intact islets from pig pancreases. The selection of the dissociation technique seems to depend mostly on the age and consistency of the donor tissue (see previous section). While the automated digestion-filtration method seems to provide excellent isolation outcomes when pancreases from suitable retired breeder pigs are dissociated [56, 77, 100] its mechanical action seems to be too harsh for pancreases from market age pigs.

Therefore, a non-traumatic static digestion procedure was established for young donor pigs [73] resembling on early attempts to isolate islets from human pancreases [99]. These methods use a closed or open container for pancreas incubation at 37°C. Mechanical dissociation is performed at later stages of the enzymatic digestion using forceps and scissors to tear predigested tissue apart. Because these digestion procedures are characterized by a fixed endpoint they are bearing the significant risk for under- as well as overdigestion. Moreover, as discussed in the previous section, the prolonged exposure of islets to anoxia [79], hyperosmolarity and harmful endogenous enzymes [80-82] during digestion can significantly reduce viability and function of islets. The early recovery of released islets from this environment seems to be absolutely essential to preserve morphological and functional integrity of islets [101]. An elegant prospective study, comparing the digestion of juvenile pig pancreases that were longitudinally splitted into two identical portions of the splenic lobe, clearly demonstrated the superiority of the automated digestion-filtration method when compared to a static manual digestion procedure [102].

Inspired by an early version of a digestion-filtration chamber, designed by Scharp et al. for digestion of the pancreas from rhesus monkeys [103], we developed a preliminary model of an oxygen-flushed chamber in order to minimize the traumatic shear stress without completely omitting mechanical treatment of the sensitive pig pancreas. As shown in Figure 2 the enzyme-distended pancreas is loaded into a basket made from a 500 µm-pore-sized mesh fixed in the center of a continuously oxygen-flushed chamber. To obtain a significant agitation of the pancreas an oxygen flow of 300 – 500 mL/min is used.

Overpressure is released through a vent in the top of the chamber. During the recirculation and collection phase the chamber is continuously perfused in a reversed direction from its top to its conical bottom supporting sampling and collection of released islets by gravity. Initial pilot studies indicated an effective release of islets with fully preserved morphology (unpublished data).

Figure 2. Continuous digestion-oxygenation device.

PORCINE ISLET PURIFICATION

Separation of isolated islets from exocrine cells, lymph nodes, vascular and ductal components of the pancreas is essential prerequisite for subsequent transplantation of islets into patients with type 1 diabetes. Islet purification is essentially needed to decrease the tissue volume which is important to limit the portal pressure [104-106] and the risk of portal vein thrombosis [107, 108] after intraportal islet infusion in the recipient. Moreover, the elimination of non-islet tissue reduces the immunogenicity of the islet preparation [109, 110] and enables successful islet culture [111-113] which is required to perform islet quality assessment and patient management prior to transplantation [114, 115].

In several animal models it has been clearly shown that exocrine tissue induces necrosis and impairs engraftment of syngeneic or autologous islets when transplanted under the kidney capsule, into the liver or into the intramuscular site [116-118]. These observations were confirmed after intraportal autotransplantation of human islets [119] and may be even more relevant for islet xenotransplantation where implanted tissue provokes a stronger response than after autologous and allogeneic islet transplantation [120-122].

The principle of separation of different cell populations is based on the tissue-specific density and on the diameter of isolated cells or tissue fragments [123]. While it might be possible to control the size of digested pancreatic tissue in highly standardized donors such as inbred mice [124], in porcine and human donors the diameter of islets and the size of digested exocrine tissue fragments vary enormously from one preparation to the other but also within the same preparation. Research on islet purification techniques has therefore been mainly focused on media and techniques that are most efficient to separate islets from non-islet tissue according to the tissue-specific density.

Determinants of intrinsic pig pancreas density:

It is obvious, that isopycnic separation of heterogeneous cell suspensions to obtain homogeneous cell populations can only successfully work when a difference exists between the density of exocrine particles and isolated islets. In fact, the specific density of acinar and islet cells differs to a small extent [125] but can be altered in the pig by several factors. One of the most important variables that influence the specific density of the exocrine part of the pancreas is the nutritional status of the donor pig. During one hour of stimulation, islets can secrete a maximum of only 10% of the initially stored insulin [126], while acinar cells can discharge more than 40% of the exocrine zymogen granules [127-129]. Because of their high molecular weight any change in the intracellular protein level has a strong impact on the density of exocrine tissue [123, 130]. Vice versa, during fasting acinar cells discharge only a marginal amount of enzymes and have a higher density compared to postprandial conditions [131]. These findings may justify the advice to retrieve pancreases only from donor pigs after overnight fasting prior to pancreas procurement.

On the other hand, any incident that causes oedematous cell swelling simultaneously reduces the density of exocrine tissue particularly after prolonged cold ischaemia time, after pancreas perfusion with large volumes of organ preservation media and subsequent to enzymatic pancreas digestion [132, 133]. The significant overlap of the specific densities of exocrine and islet tissue deteriorates the successful purification of isolated islets.

Reversing cell swelling:

Oedematous cell swelling is a phenomenon that occurs particularly at hypothermia when the sodium-potassium pumps are arrested and the membrane permeability for sodium and potassium is increased [134]. This phenomenon may vary between different tissues but can be observed in all cold-stored organs [135]. Moreover, cell swelling may continue or even increase after rewarming of the tissue [136].

It was clearly demonstrated in canine and later in human pancreases that cold storage-related cell swelling can be reversed by washing and incubation of the pancreatic digest in UW-solution thereby maintaining or enhancing the difference in the specific density between exocrine tissue and islets [137-139]. The principle of incubating digested pancreatic tissue in UW-solution prior to density gradient centrifugation was confirmed and established for purification of isolated pig islets [37, 140]. As a further development, van der Burg et al. utilized UW-solution as medium for all steps of pig pancreas processing including collagenase perfusion, pancreas digestion, and purification [69].

Due to the complexity of the UW-solution, several attempts have been made to identify which components are essential for prevention and reversion of cell swelling in order to create a simplified version of UW-solution [71, 73]. It was found that hydroxyethyl starch, lactobionate and raffinose are the most important compounds for successful purification of human and pig islets [139-141].

Selection of density gradient media:

Numerous density gradient media have been assessed for their suitability to separate islets from non-islet tissue [123]. The characteristics and efficiency of density gradient media are determined by viscosity, intrinsic density, osmolality and pH. The finding that Ficoll is contaminated by high concentrations of ions and osmotically active sucrose polymers [142] stimulated the development of iodinated density gradient media that are non-ionic, iso-osmotic and metabolically inert thus improving the posttransplant function of purified islets [143]. The ionidation of media provides a high density without increasing the osmolarity and viscosity. While the latter variable correlates with centrifugation time, the former determines the buoyant density of the separated cell populations reducing the intracellular water content by means of osmotic pressure [144, 145]. It can not be excluded that the pH of a density gradient medium also influences the volume of cells [146].

Currently, isolated human and porcine islets are purified utilizing either Ficoll-sodium-diatrizoate (Biocoll) [77, 96] or iodixanol [147, 148] as density gradient media. Apart from a higher efficiency to recover islets after density gradient centrifugation [149], iodixanol has the advantage to reduce cytokine and chemokine release from purified human islets when compared to Biocoll. This feature facilitates higher islet survival during pre-transplant culture [150]. In contrast to human islets which can most efficiently be purified when density gradient media are adjusted to a high osmolarity ranging between 400 and 500 mosm/L [151, 152], bovine, canine and porcine islets can successfully be separated from exocrine tissue by means of iso-osmotic density gradient media [143]. Recent studies have established a nearly iso-osmotic UW-Biocoll density gradient for the purification of human islets that seems to have a larger purification capacity than the Biocoll density gradient [153, 154]. However, according to the best of our knowledge this UW-Biocoll gradient has not been tested for the purification of isolated porcine islets yet.

Density gradient centrifugation technique:

Compared with rodents, purification of large volumes of digested tissue, as obtained after human or porcine pancreas digestion, is time consuming when performed in tubes, flasks

or other centrifuge vessels [155]. In order to optimize and accelerate the purification of human islets, the Cobe 2991 cell processor was established as centrifugation device for processing large tissue volumes [156]. Since its introduction in 1989 the Cobe 2991 has been established worldwide as standard device for human islet purification [97]. Moreover, the feature of the Cobe 2991 to enable loading of different density gradient media during centrifugal spinning, offered the option to manufacture large-scale linear continuous density gradients which additionally expanded the capacity to purify large volumes of tissue in one single run [157]. Unfortunately, the Cobe 2991 was originally designed to process blood cells at ambient temperature. If cooling during islet density gradient centrifugation is intended, additional significant investments for a cooled room or a custom-made cooling device have to be made [158, 159]. However, whether these technical modifications of the Cobe 2991 are essentially required for successful purification of pig islets can be questioned. In accordance with Chadwick et al. we found that pig islets can be successfully separated from exocrine tissue at room temperature [23, 160].

With respect to the high acquisition costs for a Cobe 2991, attempts were undertaken to re-establish density gradient centrifugation performed in individually loaded flat-bottom flasks [161]. Using this technique for porcine islet purification it was found that the utilization of the Cobe 2991 resulted in significantly higher islet fragmentation and lower post-purification recovery when compared to centrifugation in top-loaded flat-bottom flasks. These observations were related to the higher shearing stress being present in the Cobe 2991 [162]. Nevertheless, the data of Shimoda et al. are in contradiction to findings we previously made in the pig. Although we found an identical islet recovery after purification, the purity estimated by visual assessment, measured by amylase recovery or islet volume-to-DNA ratio was significantly higher after pig islet purification in the Cobe 2991 [37].

In this context it should be stressed that Shimoda et al. utilized a mixed discontinuous-continuous iodixanol density gradient while we have applied neutral density separation as a simple but effective and reproducible approach to purify pig islets according to their buoyancy. To perform neutral density separation which is centrifugation on one layer of iso-osmolar density gradient medium, pig islets are dispensed in 400 mL of cold iso-osmolar Biocoll of a density of 1.082 g/L which is loaded by gravity into a non-activated Cobe 2991. During spinning at 800 x g a volume of 150 mL culture medium is top-layered onto the Biocoll and centrifuged for 5 min at room temperature. Subsequently, 30 mL-fractions are collected in transparent 50 mL-culture flasks for visual inspection by an

inverted phase contrast microscope. Purified islets are pooled, washed and suspended in supplemented culture medium [77, 143].

Isokinetic islet purification:

Although neutral density purification can efficiently separate exocrine tissue from isolated pig islets, ductal, vascular and lymphoid tissue can still contaminate the final islet preparation leading to enhanced rejection of the islet graft [109]. To remove non-islet tissue of a similar density to isolated islets, it was necessary to introduce an additional centrifugation step subsequent to density gradient purification. To facilitate separation of particles according to size a linear isokinetic Biocoll gradient was procured in tubes in order to enable sedimentation at a constant and slow velocity [163]. After spinning at low speed for 90 seconds islets were collected from a discrete zone that could be visually distinguished from non-islet tissue fragments. Islets subjected to additional isokinetic centrifugation survived significantly longer in non-immunosuppressed C57/Bl6j mice when compared to conventionally purified pig islets [164].

PORCINE ISLET CULTURE

The primary aim of islet culture is to facilitate survival of isolated islets in vitro in order to pool islet preparations from different donors to provide the critical islet mass for successful xenotransplantation into patients with type 1 diabetes and to enable islet quality assessment prior to transplantation. The present review will focus on islet free-floating culture and is not considering approaches to enhance survival by embedding islets in a matrix or to reduce islet immunogenicity by different culture techniques.

Selecting the most suitable culture medium:

Most of the commercially available media were developed to support expansion and viability of different cell lines [165]. As isolated islets are not proliferating any comparison of different culture media is difficult and has to focus on recovery, viability and function of initially incubated islets. Only very few attempts have been undertaken to identify a medium that is most suitable for successful islet culture. We are aware about only two prospective studies which compare the efficiency of different commercially available culture media to promote function of cultured pig islets. However, no data regarding islet survival were provided in these studies. In one of these studies, Ham's F12 was found being most suitable for pig islet function when compared to CMRL 1066, RPMI 1640 and

TCM 199 [166]. The second study identified TCM 199 as the only medium that maintains glucose responsiveness of freshly isolated pig islets during culture because of its content of ATP, AMP and xanthine as precursor for ATP synthesis [49]. The assumption that the complete loss of peripheral alpha cells during pancreas digestion is the main cause for the non-responsiveness of pig islets toward glucose was confirmed in a subsequent study. Supplementation of CMRL 1066 with glucagon re-established glucose-stimulated insulin release of cultured pig islets [50]. These observations clearly underline that the preservation of morphological integrity during enzymatic pancreas digestion is of significant importance for pig islet function.

Glucose concentration:

Because of the complexity of culture media it is difficult to identify the beneficial compounds that made Ham's F12 or TCM 199 to the most efficient media for pig islets. The substance that represent the most important fuel for islet metabolism is glucose. In a prospective study we compared islet survival and in vitro function of pig islets cultured in CMRL 1066 supplemented with different glucose concentrations. It was found that a glucose concentration of 11.1 mmol/L increases pig islet recovery after 8 – 10 days of culture but reduces islet viability and insulin secretory capacity compared to 5.5 mmol/L glucose [167].

Serum content:

Serum is a complex and abundant source of vitamins, minerals, amino acids, lipids, hormones, enzymes and growth factors. Serum can be collected from bovine, equine, human and porcine sources and is characterized by a significant batch- and species-dependent heterogeneity [165]. While a proportion of 10% fetal calf serum is the standard supplementation for culture of rodent islets, serum has mostly been banned for clinical purposes to prevent transmission of pathogens [168]. Nevertheless, recent studies demonstrate that serum is an essential nutritive supplement to optimize survival and function of human islets particularly during prolonged periods of culture [169, 170]. This observation seems to be even more relevant for cultured pig islets which are characterized by a significant and rapid loss within a short period of time when serum-free medium for clinical purposes is used [171]. A previous study also found that certain serum replacement products do not have the same potency of serum to preserve the integrity of pig islets during culture [172]. Moreover, because of their demanding nutritive requirements long-term cultured pig islets seem to prefer the supplementation of

homologous porcine serum when compared to xenogeneic bovine serum [173, 174]. In order to further optimise pig islet survival during culture for research purposes we increased the proportion of porcine serum in culture medium to 20% [77].

Glutamine supplementation:

Another culture supplement that is used for nearly all cultured cells and tissues is L-glutamine [165]. This essential amino acid does not only serve as precursor for protein synthesis but is playing an important role as major fuel to cover the basal energy consumption of islets [175]. L-glutamine has a sparing effect on utilization of other endogenous nutrients [176] without increasing the oxygen consumption and without stimulating the insulin release [177, 178]. Because the synthesis of proinsulin accounts for a substantial proportion of islet energy consumption, L-glutamine contributes significantly to save and maintain the endogenous energy stores of cultured islets [179].

Unfortunately, the stability of L-glutamine is low. Depending on the conditions such as temperature or pH, the non-enzymatic degradation of L-glutamine in cell-empty medium can exceed 50% within 4 days of storage at 37°C [180, 181]. If proliferating cell lines are present the loss of L-glutamine can reach 90% within 4 days of culture [182]. For that reason stable glutamine compounds were introduced as supplements for culture media. However, our studies revealed a significant drop in the recovery of cultured pig islets when L-glutamine was replaced by the stable glutamine compound N-acetyl-L-alanyl-L-glutamine. Moreover, pig islets precultured in free L-glutamine were significantly more resistant toward treatment with proinflammatory mediators than islets precultured in the stable glutamine compound [183]. Because of its beneficial effects on cultured pig islets the concentration of L-glutamine has been increased to 5 mmol/L in our current culture protocol. Previously, several companies replaced N-acetyl-L-alanyl-L-glutamine by adding L-alanyl-L-glutamine or L-glycyl-L-glutamine as stable glutamine supplements. So far, no studies have been undertaken to compare the suitability of these dipeptides with free L-glutamine for human or porcine islet culture.

Culture temperature:

The culture temperature is the main determinant for the metabolic demand of cultured cells. According to the Q10 temperature coefficient any reduction of the environmental temperature by 10°C reduces the metabolic rate of organisms and cells by a factor of approximately 3 or more [184]. The reduced metabolic activity of pig islets long-term cultured at a temperature of 22°C is reflected by a two- to five-fold lower basal insulin

production and by a two- to three-fold decreased insulin stimulatory capacity when compared to islets cultured at 37°C [174]. Because hypothermia mainly affects the mitochondrial pathways of glucose breakdown the demand for oxygen is also strongly reduced [185]. As a consequence, lower temperatures significantly decrease the extent of central necrosis which correlates with a higher pig islet recovery [174]. A further reduction of the culture temperature to 4 or 1°C seems to extend the survival rate of pig islets additionally when compared to culture at 22°C [186].

Nevertheless, in spite of improved recovery, pig islets cultured at 22°C are characterized by a significantly lower viability and peripheral disintegration of islet morphology [174] which is in agreement with observations in hamster islets cultured at 37°C or 24°C [187]. From these observations the question arises whether the higher recovery of islets after 22°C-culture reflects mummification of dying islets rather than preservation of fully functional and viable islets. In fact, the Leiden group clearly demonstrated that an aliquot of freshly isolated pig islets failed to reverse hyperglycemia in diabetic nude mice while the small proportion of islets surviving after one or two weeks of culture at 37°C completely restored normoglycemia in the recipients [188].

SUMMARY

This overview has highlighted that the optimisation of currently available enzyme blends facilitates the successful isolation of islets from the pancreas of adult donor pigs. The data of the reviewed studies seem to favour a less traumatic dissociation of the pancreas particularly in younger pigs in order to maintain the morphological integrity of released islets. Pig islets can be efficiently purified using iso-osmotic ionidated density gradient media. Morphological integrity is an essential prerequisite to culture pig islets for several days to perform islet quality assessment and to pool islet preparations from different donors to provide the critical islet mass for successful xenotransplantation into patients with type 1 diabetes. The preservation of the morphological and functional integrity of isolated pig islets during culture seems to be supported by the supplementation of culture media with a large proportion of pig serum, a glucose concentration higher than 5.5 mmol/L and a high concentration of L-glutamine.

However, apart from the discussion of the technical details and prerequisites for a successful isolation of pig islets the selection of a suitable pig strain is of overwhelming importance for success and failure of any single step within the procedure of pancreas processing for islet isolation.

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