TRANSPLANT INTERNATIONAL

Transplant International ISSN 0934-0874

ORIGINAL ARTICLE

Portal Venous Oxygen Persufflation of the Donation after Cardiac Death pancreas in a rat model is superior to static cold storage and hypothermic machine perfusion

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Keywords
donation after cardiac death, islet, oxygen persufflation, pancreas.

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Conflicts of interest
The authors have declared no conflicts of interest.

Received: 27 November 2013
Revision requested: 28 December 2013
Accepted: 11 March 2014
Published online: 12 April 2014

doi:10.1111/tri.12313

Summary

Success of clinical pancreatic islet transplantation depends on the mass of viable islets transplanted and the proportion of transplanted islets that survive early ischaemia reperfusion injury. Novel pancreas preservation techniques to improve islet preservation and viability can increase the utilization of donation after cardiac death donor pancreases for islet transplantation. Rat pancreases were retrieved after 30 min of warm ischaemia and preserved by static cold storage, hypothermic machine perfusion or retrograde portal venous oxygen persufflation for 6 h. They underwent collagenase digestion and density gradient separation to isolate islets. The yield, viability, morphology were compared. *In vitro* function of isolated islets was compared using glucose stimulated insulin secretion test. Portal venous oxygen persufflation improved the islet yield, viability and morphology as compared to static cold storage. The percentage of pancreases with good *in vitro* function (stimulation index > 1.0) was also higher after oxygen persufflation as compared to static cold storage. Retrograde portal venous oxygen persufflation of donation after cardiac death donor rat pancreases has the potential to improve islet yield.

Introduction

Pancreatic islet transplantation is a therapeutic option in carefully selected patients with type 1 diabetes [1]. Successful islet transplantation depends on the transplantation of sufficient mass of viable islets and minimizing early islet loss. Two to three donor pancreases are usually needed to achieve insulin independence in these patients [2]. This demand for organs can lead to direct competition with whole organ pancreas transplant programs. Donor pancreas availability for islet transplantation programs can be greatly improved by utilizing pancreases from marginal donors, which are not routinely used for whole organ pancreas transplantation.

Islet yield from donation after cardiac death donor (DCD) pancreases with short warm ischaemia times is as good as donation after brain death (DBD) pancreases [3]. Islet yield is significantly decreased in DCD pancreases with warm ischaemia longer than 30 min [4]. Static cold storage is the current standard for the preservation of pancreases before islet isolation. Portal venous oxygen persufflation (VOP) is a novel preservation technique that has been reported in liver, kidney and heart preservation [5–7]. Recently the use of antegrade arterial oxygen persufflation of porcine and human pancreas has been reported with encouraging results [8,9]. There have been no studies so far investigating retrograde VOP as a method of pancreas preservation. This study was aimed at investigating the role of VOP in the preservation of DCD pancreases using a rat model.

Materials and methods

Rat pancreas retrieval and preservation

Male Wistar rats were used as pancreas donors. The entire recovery procedure lasted 60 min. The animal was killed by

a Schedule I method (knock on head and cervical distraction). The first 35 min of the procedure that involved most of dissection, mobilization of pancreas, cannulation of aorta, portal vein and common bile duct was termed the warm phase. During this period, the peritoneal cavity of the animal was continuously irrigated with warm saline at 37 °C. Sterile swabs soaked in warm saline covered the pancreas to keep it moist and warm.

The cold phase lasting 25–30 min, started with cold UW flush into the abdominal aorta. After the initial flush, the aorta was continuously perfused with cold UW until the pancreas recovery was completed and the organ was transferred to a petridish. During the cold phase of dissection, the surface of pancreas was once again surface cooled using sterile swabs soaked in ice-cold saline. Pancreas, duodenum and spleen were retrieved *enbloc* to minimize direct handling of pancreas. A segment of the abdominal aorta, portal vein and bile duct was retrieved along with the pancreas to enable aortic perfusion, portal venous oxygen persufflation and collagenase injection, respectively.

On the back-table, the pancreas was gently perfused through the aorta cannula with cold UW and any leaks were meticulously sutured. Pancreas was finally flushed through the aortic cannula with superoxide dismutase (SOD) solution.

Pancreases were allocated to preservation by static cold storage (SCS), hypothermic machine perfusion (HMP) or retrograde portal venous oxygen persufflation (VOP). Pancreases allocated to the SCS arm were stored for six hours in UW solution at 4 °C. HMP pancreases were perfused at 4 °C with cold UW solution through the aortic cannula at a perfusion pressure of 10 mmHg. VOP was achieved by persufflation of the pancreas with 100% oxygen through the portal vein. Small punctures in the spleen using a 24G hypodermic needle were used to vent the gas from the system. The initial persufflation pressures was adjusted to around 10 mmHg. After 6 h of preservation, pancreases underwent islet isolation. Pancreases retrieved from rats without significant warm ischaemia served as positive controls.

Islet isolation

Ice-cold collagenase solution (10 ml of 1 mg/ml Collagenase P in HBSS) was injected into the bile duct cannula after clamping the duodenal end of the bile duct. The uniformly distended pancreas was bluntly separated from the duodenum and the spleen and incubated in a static water bath at 37 °C for 50 min according to manufacturer's instructions. The tube was shaken for 10 s to homogenize the pancreas. The digest was washed thrice by centrifugation at 300 g for one minute. The digest was then passed through a 400 μ sieve to remove the undigested pancreatic tissue, lymph nodes and fibro-fatty tissue. Islet isolation from the digest was achieved by discontinuous density gradient separation using Histopaque (1.083 gm/ml). Separated islets were collected and washed by gentle centrifugation. The purified islets were suspended in 10 ml of sterile islet culture medium and transferred to a gas-permeable culture flask. The flask was incubated overnight at 37 °C with 5% carbon dioxide.

Islet counts, viability and *in vitro* assay

Measured aliquots of islets were stained with dithizone and counted. An eyepiece graticule was used to size islets. An islet measuring 150 μ in diameter was considered as one islet equivalent (IEQ). Islets of various sizes were counted separately and the final IEQ of the isolate was calculated using conversion factors for each islet size as previously described [10]. For example, 6 islets of diameter between 50 and 100 μ are together considered as one IEQ. On the other hand, one islet of 250–300 μ diameter is considered as 6.3 IEQ. The islet isolation index is a measure of fragmentation of islets. It was calculated for each isolate by dividing the islet equivalents by the actual islet count. Islet viability was assessed using propidium iodide and trypan blue staining. The viability was expressed in terms of percentage of viable islets.

Assessment of islet *in vitro* function was done by static glucose stimulated insulin secretion assay. After initial stabilization in low glucose medium, aliquots of islets were incubated in either low or high glucose medium. After three hours, the supernatant fluid in each well was collected by centrifugation and assayed for insulin concentration using a high range Rat insulin ELISA kit (Mercodia AB, Sweden). Stimulation index was calculated as the ratio of insulin secretion at high glucose concentration to the insulin secretion at low glucose concentration as previously described [1]. A SI greater than one indicated an augmented insulin secretion in response to glucose stimulation while an SI less than one indicated loss of this response [11].

Statistical analysis

Minitab™ was used to calculate the sample size required to reject the null hypothesis that there was no difference in islet yield between the three preservation methods. A sample size of 10 animals per treatment group was required to reject the hypothesis with a power of 80% at a 5% level of significance. Data were presented as mean with standard deviation. Comparison of islet yield, viability, isolation index and insulin stimulation index between the various groups was done using Kruskal Wallis test.

Results

Forty-six pancreases underwent islet isolation and purification. 12 islet isolations were from pancreases retrieved from rats immediately after death (Control group). The remaining 34 isolations were from pancreases retrieved from rats after 35 min of warm ischaemia and preserved for 5 h by static cold storage ($n = 12$), hypothermic machine perfusion ($n = 11$) or PVOP ($n = 11$).

Control group versus DCD group

As a group, islet isolates from DCD pancreases were of poorer quality as compared to control group pancreases. DCD islets were lesser in number, more fragmented with lesser viability and *in vitro* functionality (Table 1).

Comparison within DCD preservation groups

Islet number & IEQ
Static cold storage pancreases had the lowest islet numbers and IEQ, islets isolated from VOP pancreases had the highest yield in terms of numbers and IEQ. The difference was statistically significant.

Islet morphology& islet isolation index
Islet morphology showed prominent differences in three preservation groups (Fig. 1). Islets from control group were rounded and well-circumscribed. Islets isolated from SCS group were small, irregular and fragmented. Their appearance was improved in the VOP group where the islets were more rounded and large. The islet isolation index was lowest for static cold storage group and highest for the VOP group. The difference in the isolation index between the three preservation groups was not statistically significant.

Islet viability
Islet viability based on trypan blue exclusion staining and propidium iodide staining gave comparable results with good correlation. VOP group had improved viability as compared to HMP and SCS. The difference was not statistically significant.

In vitro function
The mean stimulation index for the control group pancreases was higher than the stimulation indices for the non-heart-beating-donor pancreas groups. Within the non-heart-beating-donor preservation groups, static cold storage group had the lowest mean stimulation index. There was no significant difference in the stimulation indices of the three non-heart-beating-donor preservation groups. Static cold storage group also had the lowest percentage of islet isolates with stimulation index > 1.0.

Discussion

Warm ischaemia is highly damaging to islets. Studies have shown that warm ischaemia longer than 30 min leads to > 50% loss in islet yield [4] and deleterious effects on islet viability and *in vitro* function [11,12]. In the clinical setting, it means that a significant proportion of pancreases will not provide islet yield sufficient for transplantation. In addition, warm ischaemia also injures the surviving islets leading to their increased loss after transplantation. Preservation techniques that can ameliorate the damage caused by warm ischaemia and provide an opportunity for damage control and tissue repair are needed.

VOP is a means of directly providing gaseous oxygen to the organs through the vascular system. While accidental persufflation during machine perfusion of kidneys was reported by Flatmark *et al.* in 1975 [13], it was Rolles *et al.*, 10 years later, who specifically studied this technique in pig kidneys. A subsequent clinical trial using paired DCD kidneys showed some benefit of this preservation technique [6,14]. Minor *et al.* have studied the utility of this technique in liver preservation and have reported encouraging results [15,16]. A clinical trial of DCD livers preserved by VOP also showed promising results [5]. Recently, Scott

Table 1. Comparison of islet parameters of control group and three preservation groups of DCD pancreases recovered after 35 min of warm ischaemia.

Islet variable	Control group ($n = 12$)	Static cold storage (12)	Hypothermic machine perfusion (11)	Venus oxygen persufflation (11)	*P* value
Islet count	400 (275)	173 (63)	173 (143)	254 (147)	0.045
IEQ	1042 (639)	290 (246)	369 (288)	705 (451)	0.008
Islet index	2.8 (2.3)	1.6 (1.3)	2.3 (1.3)	2.6 (0.9)	0.105
Viability	90% (9%)	74% (7.7%)	78% (12%)	81% (6.4%)	0.067
Stimulation index	1.8 (3.2)	1.2 (0.9)	1.4 (1.3)	1.5 (1.4)	0.714
Isolates with SI > 1.0	100%	55%	100%	88%	0.215

P value is for comparison between the three DCD preservation groups.

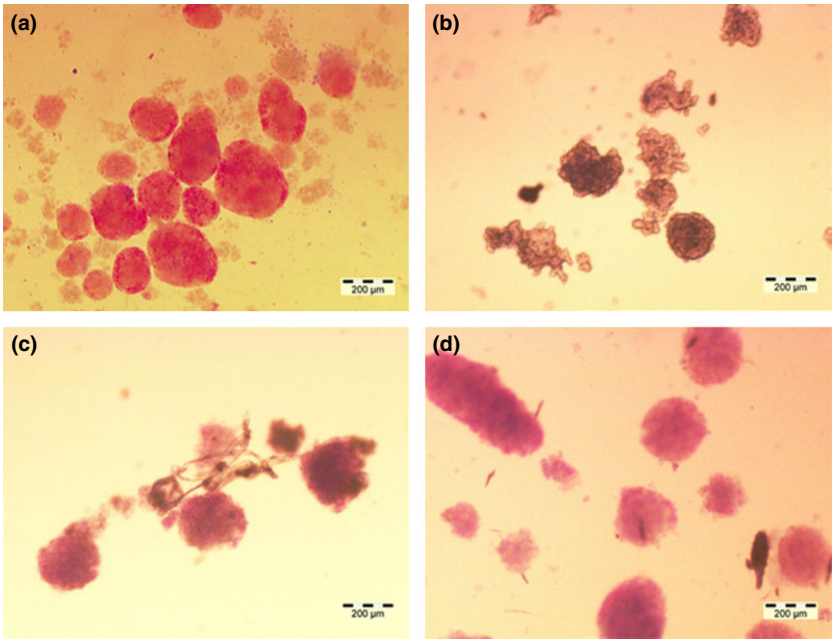

Figure 1 Representative morphology of islets retrieved from positive control and non-heart-beating-donor pancreases. (a) Control group, (b) Static cold storage group, (c) Hypothermic machine perfusion, (d) Venous oxygen persufflation.

et al. [8,9] have reported the use of antegrade arterial oxygen persufflation (AOP) as a means of preserving porcine and human pancreases. They reported improved levels of tissue ATP using MR spectroscopy in pancreases preserved by arterial oxygen persufflation. Biopsies from preserved pancreases also showed decreased autolysis in the oxygen persufflation group. However, the impact of this preservation technique on islet yield has not been reported.

AOP has the theoretical disadvantage of needing higher persufflation pressures, which can increase the risk of endothelial injury [17]. Previous studies using AOP in kidneys have shown increased vascular resistance and decreased renal blood flow in kidneys preserved with AOP. Using AOP in the pancreas is further complicated by its dual arterial supply and delicate nature of this organ. The disadvantage of retrograde persufflation is that punctures need to be made in the organ to allow the oxygen to escape from the surface. This has been reported to cause minor complications in the clinical setting [5].

Pancreas is rich in autolytic enzymes and hence is theoretically at increased risk of oxidative injury. We hence restricted the initial persufflation pressure to 10 mmHg. The pressure gradually decreased spontaneously over the preservation period. Punctures in splenic capsule were used for decompression. Punctures in the pancreas were avoided to minimize the risk of leakage from the ductal system during collagenase injection. As described by Minor *et al.* the final flush prior to start of cold preservation included superoxide dismutase to act as a free radical scavenger [18].

Mass of islets transplanted is a key criteria for the success of islet transplantation. Studies have shown that islet with better morphology (round without fragmentation) are more likely to have better viability parameters and function [19,20]. Our study has shown a clear improvement in IEQ yield when pancreases were preserved by VOP. Several possible mechanisms may explain the improved islet yield in VOP preserved pancreases. The oxygen provided by VOP may facilitate production of energy rich substrates that enable repair of warm ischaemic damage.[16] While we had not measured ATP levels in the pancreases after the preservation period, increased ATP levels after VOP have been reported in studies of liver and pancreas persufflation [9,21,22]. We also postulate that VOP preserves the glandular architecture including the ductal system enabling the uniform exposure of the entire pancreatic parenchyma to collagenase. This follows our consistent observation that VOP pancreases distended more uniformly with collagenase than SCS or HMP pancreases. This will lead to improved contact of acini with collagenase, better pancreatic digestion and more complete release of islets. It is well-known that current techniques of islet isolation from pancreases isolate only 10–30% of total islets [23]. Hence, any improvement in the fraction of islets isolated will lead to a significant increase in islet yield.

While islet morphology, viability, isolation index and *in vitro* function (stimulation index) of VOP pancreases was better as compared to SCS and MP groups, the difference did not reach statistical significance. The study

was powered to identify a change in islet yield and was probably underpowered to look at viability and *in vitro* function. Secondly, it is possible that fragmented and damaged islets from the SCS or HMP pancreatic digest are preferentially lost during density gradient separation. This may leave less fragmented, better-preserved islets in the purified fraction and can explain the nonsignificant differences in viability and *in vitro* function in the three groups.

The drawback in our study is that the rat pancreas model may not be appropriate for persufflation studies as it is thin and oxygen diffusing from the surface may negate any benefit of direct oxygenation of the graft. Studies have reported good penetration of oxygen and satisfactory ATP levels in rat pancreases preserved with static cold storage or two layer method. Persufflation is ideally tested in porcine or human pancreases where penetration of oxygen into the core of the pancreas is very poor with standard preservation techniques [24]. We had planned our study using the rat pancreas model as reliable access to large animal pancreases was not available to us. Despite this caveat, our study has shown improved islet yield and morphology of islets isolated from persufflated pancreases. Future studies of retrograde VOP for pancreas preservation should utilize large animal models. Accurate assessment of islet function will also need the use of *in vivo* testing using the SCID diabetic mouse model of islet transplantation before clinical trials are attempted.

To summarize, we compared the yield, quality and *in vitro* function of pancreatic islets isolated from rat pancreases, which had suffered 35 min of warm ischaemia and then preserved by three different preservation techniques. We found a 60% increase in IEQ yield with retrograde portal venous oxygen persufflation, which was statistically significant. Further investigation of VOP as a method of pancreas preservation is indicated.

Authorship

All authors were involved in the design of the study, collection of data, analysis and preparation of manuscript.

Funding sources

This study was partially funded by a research grant from the Royal College of Surgeons of Edinburgh.

Acknowledgements

The authors are grateful to the technical staff at the School of Applied Sciences, University of Sunderland for their assistance and guidance.

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