SUMMARY - The collagenase digestion phase of islet isolation is variable and unpredictable. This results partly from the vagaries of collagenase itself but also from the complex effects of organ retrieval and storage on collagenase digestion. Improvements in the purification of islets by density gradient centrifugation will not result from the production of new density gradient media, but rather from the continued modification of the biochemical composition of the solvents in which established gradient media are dissolved. The challenge is to produce solutions that will minimise acinar tissue swelling without compromising islet yield and viability. It is sobering to note the low recovery of islets after 48-h tissue culture (56%), and it has to be concluded that present tissue culture techniques for human and porcine islets are inadequate. Part of the problem is that the media and techniques used for islet tissue culture were designed for single cell culture and were not formulated specifically for the culture of insulin-producing mini-organs with a high metabolic rate. Cell viability is notoriously difficult to quantify, and this is no less true for isolated islets than for other tissues. The main problem is that although it is possible to determine the viability of isolated islets by measuring specific cellular functions such as glucose-stimulated insulin release, it is impossible to compare this with the same function performed by islets within the native pancreas. For this reason, it is not possible in absolute terms to determine the effect of islet isolation on islet viability.

Key-words: islet transplantation, human, porcine, isolation, collagenase, purification, tissue culture, viability.

RéSUMÉ - Isolement, culture et évaluation fonctionnelle des îlots de Langerhans. L'étape de digestion par la collagénase de l'isolement d'îlots est variable et imprévisible, du fait d'une part de la variabilité de la collagénase mais aussi des effets complexes du prélèvement et du stockage du pancréas sur la digestion par la collagénase. L'amélioration de la purification des îlots par la centrifugation sur gradient ne résultera pas de la mise au point de nouveaux milieux pour le gradient de densité, mais plutôt de modifications de la composition biochimique des solvants dans lesquels sont dissous les milieux de gradient. Il conviendrait de produire des solutions qui minimiseront la désintégration du tissus exocrine sans compromettre le rendement et la viabilité des îlots. En constatant la faible récupération d'îlots après 48 heures de culture (56%), on peut en conclure que les techniques de culture actuelles des îlots humains et porcins sont imparfaites. Cela résulte en partie du fait que ces techniques actuelles des îlots isolés que pour d'autres tissus. Le principal problème est que, bien qu'il soit possible de mesurer la fonction insulinoregulatrice stimulée par le glucose, il est impossible de la comparer à la fonction des îlots au sein du pancréas entier. Pour cette raison, il n'est pas possible de déterminer, en valeur absolue, l'effet de l'isolement d'îlots sur la viabilité insulaire.

Mots-clés : transplantation, îlot, homme, porc, isolement, collagénase, purification, culture, viabilité.

Although there is a wealth of experimental data concerning the isolation, culture and functional evaluation of rodent and small animal islets of Langerhans, much of this information has not proven directly applicable to the large animal and human situation. This review will therefore focus on the problems posed by human and pig islet isolation, the former because human islet isolation is already a clinical reality, the latter because pig islets are likeliest to be used first for clinical xenotransplantation.

## ISLET ISOLATION

Islet isolation is critically affected by all stages that precede it, and this is particularly true for the human pancreas. Warm ischaemia is especially [1] damaging and should be avoided at all costs; similarly, cold ischaemia should be minimised. *In situ* vascular perfusion is now an integral part of the organ donation operation in many countries; and, although vascular perfusion impairs porcine islet isolation [3], a prospective randomised study from Edmonton has shown for human islet isolation that vascular perfusion with University of Wisconsin (UW) solution does not reduce post-purification islet yields as compared to no perfusion at all [4]. The question of which vascular perfusate to use for the human pancreas has not been the subject of prospective studies. However, EuroCollins solution appears to be detrimental to human islet isolation [5] and is certainly not an effective vascular flush solution if cold ischaemic time is greater than 6 h [6]. Although UW solution appears to provide reasonable pancreas preservation for up to 18 h [7] prior to human islet isolation (based on International Islet Transplant Registry data [8]), it would seem inadvisable to transplant islets isolated from UW-perfused human pancreata with a cold ischaemic time of more than 12 h.

Intraductal distention with collagenase suspended in UW solution immediately after pancreas excision allows effective storage of the pig [9] pancreas for up to 24 h prior to islet isolation. Unfortunately, this approach has provided very poor islet yields and viability in the human for both stored and non-stored pancreata [10], probably because UW solution contains inhibitors of collagenase [11] and is toxic to human tissues at 37°C [12]. The challenge in the human therefore is to develop UW-based cold storage solutions that optimise preservation when given intraductally but do not compromise islet yield or viability. It is convenient to subdivide the process of islet isolation into pancreas dissociation and islet purification.

### Pancreas dissociation

Current methods for human and pig pancreas dissociation are based on the principles described by Gray *et al.* [13] in 1984. The technique consisted of the intraductal administration of collagenase [14], followed by incubation at 39°C, with gentle teasing and shaking, and then trituration through wide-bore needles. Although improvements in this manual method have been described [15], the description of an automated method by Ricordi *et al.* [16] is generally agreed to represent a significant advance. Indeed, porcine studies [17] that have compared manual with automated methods have shown significantly increased islet yields (three times as many IEQ) with the automated method. The principles behind the automated method are minimal physical trauma and continuous collagenase digestion of the pancreas during which islets are continually removed from the injurious action of collagenase [18].

Regardless of the technique used, the collagenase digestion phase is critically dependent upon the efficiency of the collagenase. A good enzyme will release intact islets that are entirely free of exocrine tissue (cleaved). Unfortunately, batch-to-batch variation and deterioration of a good batch with storage are a common experience. Indeed, the vicissitudes of collagenase [19-21] have driven many investigators to despair, and few would disagree with Scharp [22] that collagenase is one of the major obstacles to successful human islet transplantation. Crude collagenase is derived from cultures of *Clostridium histolyticum* and may contain up to 11 different collagenases, each with different specificities towards different collagens [23]. In addition, crude collagenase contains numerous other enzymes [21]. Highly purified collagenase is ineffective for islet isolation [24], and some of the other components of crude collagenase are therefore necessary. Recent rat studies have investigated the role of the various types of collagenase present in crude collagenase on islet isolation and have shown that pancreas dissociation can be manipulated by using defined mixtures of collagenases. These studies need to be repeated in the human because it has been shown that there is a marked species variation in both total pancreatic collagen content [25] and collagen type and distribution [26]. Thus, unfortunately, a collagenase batch that works well in the pig or dog may not work in the human. The recent development of a collagenase blend called “Liberase” may represent an advance [27]; prospective randomised trials are however still awaited.

The efficiency of the collagenase digestion phase may be affected by a number of factors in addition to the collagenase batch. The pancreas should be uniformly distended because islets are not isolated from undistended segments [28]. The question as to whether the collagenase solution should be perfused or loaded into the pancreatic duct has been investigated by Warnock *et al.* [6] who showed, although not conclusively, that the ductal perfusion technique improved islet yields. A further issue is the optimum timing of collagenase injection. There is inevitably a period of cold storage between pancreas excision and...
the commencement of islet isolation. Ohzato et al. [29] have shown in the rat that cold storage leads to loss of ductal integrity, so that during ductal distention the pressures achieved were lower, pancreas distention was suboptimal, and collagenase was more likely to enter the islets themselves. It was also shown that the yields of rat islets were increased if collagenase was given intraductally in Hanks’ balanced salt solution (HBSS) at the time of pancreas excision prior to cold storage for 4 or 6 h. It has subsequently been shown that for young human organ donors (< 30 yr) intraductal injection of collagenase in HBSS prior to, rather than after, 3-h ischaemia, significantly improves islet yields [30]. It has however been shown for donors of all ages that immediate intraductal injection of collagenase improves islet survival in low temperature culture.

It can be deduced from the foregoing discussion that the collagenase digestion phase of human islet isolation remains variable and unpredictable. Indeed, it has been shown that the proportion of pancreatic insulin recovered in the pancreatic digest after collagenase dispersion of the human pancreas ranges from 7 to 91 % [15]. Much of this variability relates to the effects of cold storage and the vagaries of collagenase itself, and these issues must be considered as research priorities.

**Purification** — Although a large number of islet purification techniques, including differential sedimentation at unit gravity [31], filtration [32], the use of Velcro to remove acinar tissue [33], centrifugal elutriation [34], cryopreservation [35], gamma irradiation [36], anti-acinar cytotoxic antibodies [37], tissue culture [38], the selective destruction of acinar cells by laser energy [39] or hypotonic lysis [40], magnetic microspheres coated with anti-acinar cell monoclonal antibodies [41], and fluorescence-activated cell sorting [42] have been used in other species, density gradient centrifugation is the only technique that has been successfully used for large-scale porcine and human islet purification. The technical aspects of the discussion will therefore focus on density gradient purification. Human islet purification has proven particularly difficult because both the density and the diameter of acinar tissue, and to a lesser extent of islets, change from one preparation to another [43]. Islets normally vary in diameter from 15-500 µm and, in addition, the diameter of islets and acinar tissue is critically dependent upon the collagenase digestion stage of the isolation process. This intrinsic variation in islet diameter, combined with the effect of collagenase digestion, means that we have no control over endocrine and acinar tissue size distribution. Because of these highly variable and overlapping tissue diameters, it is not possible to purify islets from acinar tissue using velocity sedimentation, and we are forced to use isopycnic centrifugation.

There are a number of possible causes for variable acinar tissue density. First, it is possible that acinar tissue density depends on the secretory status of the acinar cells. Second, the density of acinar cells may be affected by the size of the aggregates formed [44] by the collagenase digestion of the pancreas, and finally, acinar tissue density may be reduced by cellular swelling and oedema. Recent studies [45] have shown that the single most important factor affecting the density of acinar tissue during islet isolation is acinar tissue swelling and oedema, acinar tissue degradation being relatively unimportant. Acinar cell swelling can be provoked by a number of insults, including mechanical trauma [46] and hypothermia, and collagenase digestion has been shown to influence cell membrane permeability and to cause cell swelling [47].

Broadly speaking, techniques for optimising density gradient purification can be divided into physical and chemical ones. We will first consider physical parameters. For cell separation to be optimised, it is essential that certain experimental conditions be controlled. Temperature may affect the results of density gradient purification [48], and it has been shown that the results of human islet purification using BSA are the same at 4 °C and 22 °C, whereas porcine islet purification is more efficient at 4 °C [49]. Continuous gradients offer many theoretical advantages over discontinuous gradients [50], and it has been demonstrated that large-scale continuous density gradients can be established on the COBE 2991 processor [51, 52]. This therefore is the preferred technique for large-scale human islet purification. It has been shown that, compared to discontinuous gradients on the COBE 2991, continuous gradients improve human islet yield by 26 % and also improve islet viability.

Chemical methods for improving the purification of human islets by isopycnic centrifugation aim to increase or maintain acinar tissue density whilst leaving islet density relatively unaltered. One approach has been described by van der Burg et al. [53] who greatly improved the results of canine islet purification by collecting and washing the pancreatic digest in UW solution prior to density gradient centrifugation. These findings have been confirmed in the human [54], and for some isolations the results of density gradient purification are further improved by storing the digest in UW solution (at 4 °C) for 1 h prior to centrifugation. The beneficial action of UW storage results largely from the presence of the extracellular impermeant anion lactobionate [45] and the colloid hydroxyethyl starch [55].

The most commonly used gradient media for human islet isolation are Ficoll [6], Euroficol [56], Ficoll-Diatrizoic acid [1] and hyperosmolar bovine serum albumin (BSA) [57, 58]. Further improvements in the purification of islets by density gradient centrifugation will not result from the production of new density gradient media, but rather from the continued
modification of the biochemical composition of the solvents in which established gradient media are dissolved. These modifications may however produce an unphysiological environment [59], and consequently their effect on islet viability must be determined. In the human, the challenge is to produce solutions that will minimise acinar tissue swelling without compromising islet yield and viability. It has been shown that hyperosmolar BSA density gradients improve human [43] (500 mOsm/kg/H₂O) and porcine [49] (400 mOsm/kg/H₂O) islet purification. This phenomenon has been reported for other cell types [60] and in the case of islet purification results from the hypertonic environment differentially increasing the density of acinar tissue more than endocrine tissue [61]. A further promising approach to minimise acinar tissue swelling is the construction of density gradients by the addition of extra hydroxyethyl starch or Percoll to University of Wisconsin solution [62].

**ISLET CULTURE**

The culture of islets poses several unique problems. First, due to the size of the islets, the central core can become necrotic, probably as a result of inadequate oxygen supply. Second, unlike many single cell types, islets do not proliferate in culture, and this makes the assessment and comparison of different culture techniques difficult. Usually, the proliferation rate of a cell line is the determinant of the effect of a change in the culture medium composition. Finally, islets from different species may have unique requirements or behaviour in tissue culture. For example, pig islets tend to fragment very easily in culture, probably due to the lack of a defined capsule [63].

It is pertinent to begin by asking “is it necessary to culture islets?” First, there is the need to store islets prior to transplantation. Second, a period of low temperature may be used to immunomodulate islets to abrogate rejection of the transplanted tissue. Third, Weber et al. [64] have demonstrated that a period in culture can be used to purify islet preparations. Although there are many positive reasons for culturing islets, the potential risk of infection should also be considered. One recent report has highlighted the problem of infected islets leading to episodes of bacteremia in islet transplant patients [65]. Although the source of this infection was determined to be cryopreserved islets, it is apparent that any increase in the number of procedures undertaken on islets will increase the risk of infection occurring. Other reports assessing the sterility of islet preparations or the incidence of infection in patients following islet transplantation have tended to demonstrate factors other than the culture of islets as having the greatest risk. For example, Scharp et al. [66] found that the greatest infection source was from the pancreas transport fluid, whilst the Ficoll density gradient medium was the major source of contamination introduced during processing.

**Commercially available media** – One of the earliest studies comparing the culture of islets in different commercially available media was undertaken using rat islets in 1978 by Andersson [67]. The study compared the effectiveness of TCM 199, RPMI 1640, CMRL 1066, MEM and Ham’s F10. The results demonstrated that the islets in F10 had the highest insulin content, but that the highest insulin biosynthesis rate was in the islets cultured in RPMI. Andersson therefore concluded that RPMI was the medium of choice for islets, possibly due to the nicotinamide content and the 11mM glucose concentration. Davalli et al. [68] reported the results of culturing porcine islets in CMRL 1066, RPMI 1640 without glucose, RPMI 1640 with 11mM glucose, Ham’s F12 or TCM 199. All the media were supplemented with 10% FCS, and the islets were tested by insulin release. Only TCM 199 gave an increase in glucose-stimulated insulin release, whilst islets cultured in either CMRL or RPMI (both with or without glucose) showed a decrease in release. The authors commented that TCM 199 is the only one of these media which contains adenosine phosphates and xanthine, and that these could therefore be of importance in islet culture. They also suggested that the relatively high glucose concentrations of Ham’s F12 (9.9mM) and RPMI could be toxic to the islets.

The results of the above studies suggest that islets isolated from different species may have different requirements in culture. This is supported by the results of Holmes et al. [69] who cultured rat, porcine and human islets for a week in ten different commercially available media, providing a more comprehensive comparison than had previously been undertaken. They concluded that Iscove’s MEM was the best medium for rat islets. Ham’s F12 nutrient mixture was best for porcine islets, and CMRL 1066 was best for human islets. These results do not concur with the above studies, although this could be due to Iscove’s medium not having been previously tested. Moreover, the length of time the islets were in culture was greater than that used for porcine islets by Davalli et al. [68] (48 h compared with a week), which could have affected the results. A recent report from Rastellini et al. [70] examined the effect of adding pyruvate (7mM) to CMRL-1066 on the viability of cultured human islets. After 60 days in culture, 67% of pyruvate-cultured islets could be recovered compared to 0% in the control group. This promising approach certainly merits further investigation.

**Serum-free media** – Foetal calf serum (FCS) or newborn calf serum (NBCS) is routinely added to culture medium for a wide range of cells and tissues. The reason for this is because the serum contains many
components which are beneficial for cell survival and growth. Unfortunately, however, there can be a considerable variation between batches of serum, some of which may even be toxic to islets. Much of the work undertaken on the effect of serum on islet culture has assessed whether it is possible for islets to survive without the presence of serum if other constituents are added to the medium. A major reason for trying to omit serum from islet culture medium is to move away from the use of animal products when tissue is to be used for human transplantation. Tai et al. [71] undertook a study on porcine islets, assessing the use of serum replacement products. They found that they were not suitable for long-term culture and suggested that this could partially be due to variation between batches of these products. These studies demonstrate that more developmental work is required before islets can be routinely cultured in serum-free medium.

Oxygenation – Dionne et al. [72] assessed the effect of oxygen concentrations on insulin release from canine islets, comparing normoxia (142 mmHg) with a range of hypoxic conditions (minimum of 5 mmHg). They found that the second phase of insulin release was decreased due to hypoxia. The authors commented that this was thought to be due to pO₂ gradients outside and inside the islets. This results in the islets being exposed to low pO₂ decreasing radially from the periphery to the core, possibly leading to depletion of the energy stores from the beta cells. It has been shown that liver cells are in oxygen debt within a few hours of establishing a primary culture if the medium is 3 mm deep. It is probable that such an effect would occur with islets under similar circumstances, and this highlights the speed at which less than optimal culture conditions can affect the viability of the tissue. It is clear, therefore, that methods of improving oxygen supply to the islets in culture, and diffusion into the centre of the islets, are required. One factor, already highlighted above, is the depth of the medium. This should be kept to a minimum to allow rapid diffusion of oxygen to the islets, and a depth of 2 mm has been recommended for cells with a high oxygen requirement.

Alternative methods may include the addition of oxygen carriers to the medium, constantly bubbling oxygen through the medium, a rolling culture system, or a stirred culture system in which the islets are maintained in suspension by a magnetic stirring bar suspended from the top of the bottle. The potential problem with any system designed to keep the islets constantly moving is the fragility of the islets, especially porcine islets. Tests would be required to determine whether this type of culture system is suitable for islets, and whether it can be applied to all species of islets.

The role of exocrine tissue – A study undertaken by Metrakos et al. [73] assessed the effect of interactions between different cell types on the survival of islets in vitro. After preparing ductal tissue from the pancreas and setting up cultures of the islets together with the ducts, they found an increase in proliferation of beta cells in the presence of the ducts compared with the culture of islets on their own. They concluded that there is an interaction between the ducts and the islets, and that this leads to an increase in proliferation, possibly as a result of the release of factors from the ductal tissue.

Culture temperature – Culture temperature has been investigated with respect to its effect both on islet immunomodulation and recovery. In 1991, Lacy and Finke [74] reported their findings on the effect of culturing rat islets at 24 °C rather than at 37 °C which had been the standard culture temperature. They found that at the lower temperature they achieved almost complete removal of the intraislet lymphoid cells, as long as CMRL 1066 was used as the culture medium. When RPMI 1640 was used, there was a considerable reduction in the number of lymphoid cells remaining, although there were still residual cells detectable. Interestingly, RPMI was originally developed for the culture of lymphocytes [75], whereas CMRL 1066 was originally developed for the culture of fibroblasts and kidney epithelial cells [75]. Recently, Dabbs et al. [76] have shown that 7-day low-temperature (24 °C) culture can slightly prolong islet allograft survival in CsA-treated dogs.

Lakey et al. [77] assessed human islet recovery after 24-h culture as part of a study on cryopreserved islets. They reported a mean recovery rate of 72.5% for islets cultured at 22 °C compared with 54.8% at 37 °C. This study indicates a considerable benefit for islets cultured at 22 °C as compared to 37 °C.

Culture matrix – One factor which also needs consideration is whether islets should be cultured in a three-dimensional matrix or be maintained as a free-floating culture. Ohgawara et al. [78] cultured foetal porcine pancreas in a collagen gel matrix. They found that nicotinamide had to be added to the culture to prevent the degradation of the collagen gel. However, the authors noted that the nicotinamide had the added benefit of preventing the growth of fibroblast like cells. Brendel et al. [79] compared the culture of human islets in standard suspension culture and in matrices of either agarose or alginate. They found that the number of islets surviving over the period of study was greater when the matrices were used, and that agarose provided better results compared with alginate. The insulin release results were also better in agarose, although the ratio of total insulin release to total DNA was not significantly different between the groups. All the islets had good perfusion responses, but the best transplantation results were obtained with the islets which had been cultured in the agarose matrix. The authors commented the ease with which the islets could be retrieved
from the gel when they were required for transplantation, a problem which can result in the loss of a lot of islets when other matrices are used. The two major problems associated with the culture of islets in three-dimensional matrices are the loss of islet tissue when they are retrieved from the matrix, and the diffusion of nutrients and waste products across the matrix. The latter study suggests that these problems may have been overcome. If this is the case, this system of culture may be of use for routine culture of islets. However, before this can happen, the other aspects of the islet physiology and viability require study, for example, the supply of oxygen to the islet and the problems of central necrosis.

Although they did not use matrices for islet culture, Hofer et al. [80] assessed the use of wellplates with inserts for the culture of islets. They found that the inserts did not allow cell attachment and therefore prevented fibroblast growth. The islets maintained good three-dimensional form and good viability. The authors also listed several advantages of this system: the endocrine tissue was of high purity; it was easier to change the medium; they could perform tests by moving just the inserts, preventing the loss of any islets; and the collection of islets was improved without causing them any damage.

Conclusions – The media currently used for islet culture were originally designed for proliferating cell lines and were not specifically designed for the culture of highly specialised metabolically active "mini-organelles". The optimum conditions for islet culture need to be determined, both in terms of the nutrients utilised by the cells and the ability to ensure their supply to core cells at the centre of the islets. Furthermore, the results of published studies suggest that the culture requirements of islets may be species-specific, and new media must be developed with this in mind. In conclusion, at the present time islets are cultured in "adopted" media, and there is clearly an urgent need to develop specific culture media for islets of Langerhans. Only then will islet culture be by design rather than by proxy!

ISLET VIABILITY

Islet viability may be adversely affected by innumerable factors including events occurring before and during organ donation, during the period of cold ischaemia prior to islet isolation, the isolation process itself, and the period of storage (tissue culture and/or cryopreservation) prior to transplantation. After transplantation, islet viability may suffer due to impaired revascularisation or the action of islet-toxic substances. All of these factors will of course increase the number of islets required to produce normoglycaemia. The impact of impaired islet viability on the outcome of human islet transplantation can be deduced from the observation that in the period 1990-1992 no islet preparations prepared from pancreata with a cold ischaemic time greater than 12 h produced insulin independence [8].

Unfortunately, cell viability is notoriously difficult to quantify [81], and this is no less true for isolated islets than for other tissues. The main problems are that, although it is possible to determine the viability of isolated islets by measuring specific cellular functions (e.g. glucose-stimulated insulin release), it is impossible to compare this with the same function performed by islets within the native pancreas, so that insulin release reflects the function of only a single metabolic pathway within islets and changes in insulin secretion in response to alterations in glucose concentration may not reflect changes in global islet function or viability. For these reasons, it is not possible in absolute terms to determine the effect of islet isolation on islet viability. It is possible however to estimate the effect of various procedures (e.g. different density gradient media or cryopreservation) on the viability of islets after isolation. The in vitro methods available include light [82] and electron microscopic morphology [83], fluorometric membrane integrity assays [84-87], colorimetric tests of mitochondrial function [88, 89] and glucose-stimulated insulin release [90, 91]. The results of islet perifusions should be interpreted with caution. Thus, cryopreserved canine islets that failed to secrete insulin during perifusion consistently induced normoglycaemia after autotransplantation [92], and it has been reported that a poor response from human islets during perifusion did not predict their in vivo function after transplantation [93]. Undoubtedly, the best index of viability is the ability of transplanted islets to reverse diabetes [94], which in the case of human islets can be achieved by transplantation into the diabetic immunodeficient mouse [95, 96] or rat [97]. The problems with these latter methods are the difficulties of maintaining immunodeficient rodents and that, although reversal of diabetes shows that the islets are viable, it does not show how viable.

In summary, although islet viability is a critical factor that determines the outcome after transplantation, there is no currently available method for standardising viability assessment. It would however seem sensible to at least confirm that human islets are not dead prior to transplantation, and membrane integrity assays or perifusions are the most convenient way of doing this at the present time.

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