Acta Scientific MEDICAL SCIENCES (ISSN: 2582-0931)

Volume 8 Issue 3 March 2024

Research Article

A Simple, Time Efficient Purification Methodology Providing Good Quality Human Pancreatic Islets in the Indian Population

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DOI: 10.31080/ASMS.2024.08.1779

Received: February 19, 2024

Published: February 29, 2024

Abstract

Recent advances in islet cell transplantation have led to insulin independence in a majority of islet transplant recipients. Numerous protocols have been published describing isolation of human pancreatic islets, but often rigorous and systematic assessment of their integrity is lacking.

We have described a simple, effective method for the isolation and purification of human pancreatic islets from the Indian population so that we can facilitate preclinical allogeneic islet transplantation studies in the Indian population. In this study, we propose 2 different patterns for purification via a simple filtration process and also via gradient technology for the optimal generation of islets. We have also assessed the functionality of the insulin producing islet cells purified by both the methods. The technique yields sufficient numbers of pure and viable islets to support preclinical research to develop improved strategies to prevent the immune destruction of the transplanted islet graft.

Keywords: Diabetes; Beta Cells; Islets; Insulin; Human Pancreas; Purification; Ficoll; Filtration

Introduction

Transplantation of normal isolated islets of Langerhans for the treatment of diabetes remains an elusive goal in clinical practice, even though it has been possible for some years to cure experimental diabetes in rodents. Beta cell destruction or insufficient insulin productions are the hallmarks of diabetes mellitus (type 1 diabetes). The up-surge in the prevalence of diabetes mellitus has led to the consideration of diabetes as an epidemic of the 21st century affecting over 246 million people worldwide and is expected to affect 380 million by 2025. Current therapeutic strategies available for treating type 1 diabetes include but are not limited to daily insulin injections combined with meticulous blood glucose monitoring and necessary dietary adaptations. Pancreatic islet cell transplantation can improve blood glucose levels, and thus proves to be a promising treatment modality for type 1 diabetes. Upcoming research should focus on its availability and reach to the needy [1].

The isolation of islets of Langerhans is a difficult problem because islets comprise only about 10% of the pancreas. The

size, architecture, and compact fibrous nature of the human pancreas, in particular, present additional problems for the islet separation procedure. This procedure should ensure the following requirements: 1) first and foremost, the action on islets is minimally traumatic, 2) secondly, there is a scope for preserving the progressively liberated islets through continuous digestion, avoiding any further enzymatic action on them, 3) the digestion process demands a bare minimum human intervention, and 4) the isolated islets should hold desirable purity and high yield. The separation and purification of large numbers of intact human islets from one donor pancreas is the first limiting factor in human islet transplantation. All of the procedures developed for the isolation of islets from large mammals, including humans, have in common a significant traumatic factor in the isolation, digestion and most importantly the purification process. Perhaps the major problem preventing clinical islet transplantation has been the lack of a method for isolation of islets from the human pancreas in sufficient numbers, relatively uncontaminated by exocrine tissue and of adequate viability [2].

The 3-step process of islet isolation commences with enzyme perfusion, followed by pancreas digestion and finally the islet purification. Islet purification is a vital step as preparations with high purity assures manifold benefits like superior safety, better engraftment, reduced graft immunogenicity in transplants, and enhanced suitability for immunomodulation procedures [2]. The density gradient centrifugation method of islet purification has been the most common practice ever since 1960. This method was established on the basis of varied densities of islets and exocrine tissue, both in rodents and humans [3-7]. Nevertheless, as years rolled by, alternative methods such us filtration or osmotic shock have also gained prominence owing to their usage of physical properties other than density [8,9]. In the recent years, histopaque has been highlighted by McCall and colleagues as a better method for furnishing healthy islets [10]. The need-of-the-hour for large-scale islet isolations is a simple and faster method, which optimizes time especially during manpower paucity. Henceforth, considering the speed, cost-effectiveness and simplicity of filtration method, it can be a good option for islet purification. Still, the islet quality yielded through filtration method needs to be thoroughly analyzed.

Methodology

Ethical approval for the organ procurement

'Research involving humans, including observational, survey-based studies, or any personal data, must have been performed by the declaration of Helsinki and must have been approved by an appropriate ethics committee', and strictly abiding by this, the ethical committee of the present study was approved by Apollo Hospitals [AMH002-04/18] and Velammal Medical College and Hospital [VMCIEC/01-2021]. The protocols were designed according to the guiding principles approved by the Indian Council of Medical Research without affecting the Indian organ procurement program. The primary importance was given to the transplant patients and if there were no takers for the organ it was diverted for the purpose of research. With due consent, pancreata were procured from adult heart-beating cadaver (humans). Informed consent was specially sort for use of pancreatic tissue in research from Organ donors and all donors' families.

Donor selection

Donor pancreas quality is an important factor in successful islet isolation [11-14]. However, pancreata are offered first for the therapeutic application and then only for islet transplantation since whole-pancreas transplantation is considered an established treatment and islet research is considered experimental. We couldn't fix any specific criteria on the selection of donor or pancreas considering the organ procurement program and the ICMR guidelines.

Pancreas retrieval

We retrieved a total of 15 human pancreata using a protocol similar to the one as whole-organ pancreas transplantation. En bloc dissection with hypothermic in situ vascular perfusion with University of Wisconsin (UW) solution along with immediate surface cooling of the pancreas was achieved with 4°C ice slush in the lesser omental sac [15-17]. Pancreas was taken by surgeons for the biopsy related studies and the remaining was given for the purpose of islet isolation. We transported the organs via road with all essential documents, as the current norms and regulations didn't allow us to airlift them. After procurement, the pancreases were shipped in cold University of Wisconsin (UW) solution or histidine tryptophan ketoglutarate (HTK) from the donor hospital

to the islet isolation laboratory maintaining the cold ischemia time in the range of 4 hours and 30 minutes to 19 hours and 47 minutes.

Islet isolation

Our group has modified and developed islet isolation protocol considering all the norms and regulations and this can be further adapted and modified to clinical protocol in a GMP facility. Extra fat, connective tissue, and blood vessels were removed and the pancreas was washed in a cocktail of antibiotics that is weighed and dissected. The exogenous enzymes such as the collagenases and neutral proteases (Serva, GMP grade, Heidelberg, Germany) were uniformly mixed, freshly dissolved in HBSS (Mediatech-Cellgro, Inc., Manassas VA, USA) and were pre-warmed at about 28 to 30°C. This infusion containing a blend of enzymes was done at 37°C, and such an infusion of enzymes usually distends the pancreas. In the Ricordi chamber, manual shaking with a solution of same enzyme blends circulates and disrupts the pancreatic tissue [18,19].

Pancreatic tissue samples were obtained every 5 to 10 minutes and digestion process was observed. To evaluate the islets, the samples are suspended in a solution of HBSS containing Dithizone (DTZ), a dye that colors the islets in red to distinguish them from unlabeled exocrine and ductal tissue [20,21] When islets free of exocrine tissue were visible, enzyme activity is terminated by cooling and the addition of Human serum albumin (HSA)/HSA with HBSS. The samples were then centrifuged twice at 1200rpm at 4^0C for 5 minutes. The supernatant was discarded and the pellet equally divided in to two ways and one was taken for purification using discontinuous ficoll gradient method and the other was taken for filtration.

Purification-filtration

The purification of islets was in accordance with the protocol set by Li., *et al*. 2009, but for a few necessary modifications (Collagenase P instead of Collagenase XI, filtering through a 100 µm cell strainer instead of a 70 µm one).

Purification-Ficoll gradient

Depending on the pellet size, the gradient mixtures were prepared. Initially, a high-density gradient [1.119] was mixed with the islet solution, this was slowly followed by carefully layering the islet solution with medium [1.083] and low-density [1.077] gradients. It was then centrifuged at 2400rpm for 20 minutes at 4^0C. The islets were separated at the interphase. The islets were decanted in to a fresh tube [22].

Islet quantification and quality control testing

A direct count of the islets stained with DTZ determines the islet yield [18]. An islet equivalent defines the volume of an islet with 150-µm diameters. Trypan blue exclusion assay was used to check the viability of islets. Islets were also assessed by MTT assay and the functionality was done using the Insulin chemiluminescence assay. To check for microbiological contamination, the islet cell samples and the procurement fluid (a fluid wherein pancreas gets preserved until isolation) are analyzed by Gram stain, and sterility culture.

MTT assay

To 100 µl of filtrate samples and Ficoll samples each, 100µl of MTT solution (0.05mg/ml) will be added. To this 100 µl of DMSO will be added and readings will be taken at 0^{th} and 4^{th} hrs at 570nm using Spectramax i3. Cell viability percentage will be calculated by using formula

Viable cells (%) = (Mean optical density value at 4^{th} hour/Mean optical density value at 0 h) x 100

Insulin chemiluminescence assay

For quantitative determination of human insulin from its tissue samples the ALPCO Insulin Chemiluminescence ELISA is best suited, and hence we purchased it. The instructions given in the kit was strictly followed. The curve was plotted for the standard concentration along with the test sample concentration in pg/ml.

Statistical analysis

All the statistical analysis was performed using Graph-pad prism (Version 6). All the data were expressed as Mean ± standard deviation (STD) and Pearson correlation was done between the measured variables with $p < 0.05$ was considered as statistically significant.

Results

Donor characteristics

The donor characteristics from 15 islet isolations are presented in Table 1. The mean age of donors was 41 years and ranged from 18 to 76 years. Cerebrovascular accidents accounted for

the most frequent causes of death. The mean CIT was 12h39m, ranging from 6h40m to 19h47m. The stay in the hospital for the majority of donors was less than 5 days. We have processed a total of 7 diabetic samples and 5 non-diabetic samples. The mean BMI ranged between 33.1 and 17.9. The mean maximum and minimum HBA1C were 8.3 and 5.2, respectively.

Biochemical Parameters	Mean	Std. dev.
Age (yrs)	41	17
Gender (M/F)	9/ 6	
Height (cms)	167.17	6.55
Weight (kg)	68.96	14.81
BMI	24.63	4.25
Diabetic/Non-diabetic	7 / 5	
HBA1C	5.92	1.03
Cold Ischemic time (hrs)	12.39	5.23

Table 1: Clinical and Biochemical characteristics of donors.

All data represented as mean ± standard deviation (SD).

Islet processing details

Details about islet processing are presented in Table 2. Considering the regulatory norms as per the government and donor organ program, the sample weights vary every time. Hence, to present the results, we have considered the yield of islets per gram of tissue. A mean digestion time of 25 to 30 minutes was observed for all the samples. The total number of viable cells per gram of the tissue for each sample is shown in Table 2.

Processing details	Mean	Std. dev.
Weight of sample processed (g)	11.06	6.47
Digestion time (min)	28	6
Total number of viable cells/g of tissue	0.47 X 10^6	0.29 X 10^6

Table 2: Islet processing details of Donors.

All data is represented as mean ± standard deviation (SD).

Isolation outcome is affected by multiple factors. Digestion of the human pancreatic tissue and its separation from acinar tissue plays a prominent role. Based on our own human islet isolation experience, we have defined the following evaluative points during the isolation process. They are,

- The digestion time is different for each and every sample.
- Once 50-60% of the islets are free and are separated from acinar tissue, add dilution solution to cool the tissue and this plays a crucial role and the process of digestion should be stopped.
- The tissues must be handled carefully while washing, mixing and also during the collection process.
- The digested mixture of pancreatic tissue should optimally be placed in ice for a minimum time of half-an-hour so that it yields better purification results.

In Figure 1 and Figure 2 we can see a clear difference between the islets that were inside the tissue and the islets that were free.

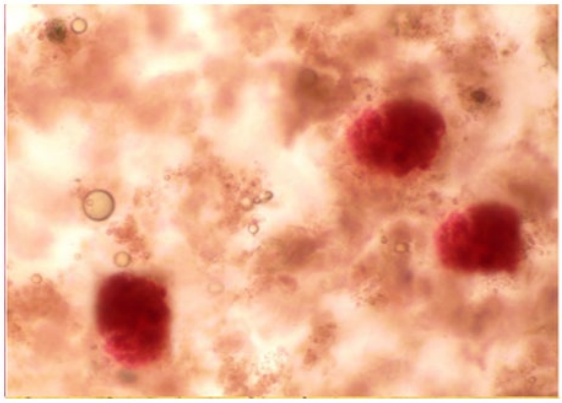

Figure 1: Microscopic image of human pancreatic islets stained with dithizone staining at 40X.

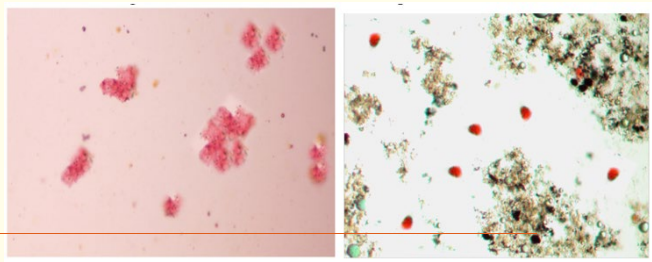

Figure 2A and Figure 2B: Microscopic image of human pancreatic islets stained with dithizone staining at 40X: Individual islets seen after purification.

The following figures illustrate human pancreatic islets stained with dithizone staining. (Figure 1), islets seen separately after 25-30mins of digestion (Figure 1), individual islets seen after purification (Figure 2A and B).

Role of clinical parameters in correlating islet yield

Interestingly, our findings stated an influence (positive/ negative) of clinical parameters on the islet yield. A positive correlation was seen between the cold ischemic time and islet yield. On the contrary, HbA1c and age correlated negatively with the islet yield. A statistically non-significant, positive correlation was observed between BMI and the islet yield in both diabetic and non-diabetic patients; this statistical non-significance could probably be attributed to the paucity in sample size [Table 2 and 3].

Parameters	Islet cells	
	r value	p value
Cold Ischemic time (hrs)	-0.1073	0.7835
HbA1c	-0.0251	0.9383
BMI	0.1264	0.6954
Age	-0.2018	0.5293

Table 3: Pearson correlation analysis of islet cells isolated with metabolic variables.

The viability of cells assessed by the MTT assay showed that the recovery rate or the percentage of cell viability for the human pancreatic islet cells from the Filtrate method were little higher than the discontinuous gradient Ficol method. The average was taken on all the samples and presented as Figure 3.

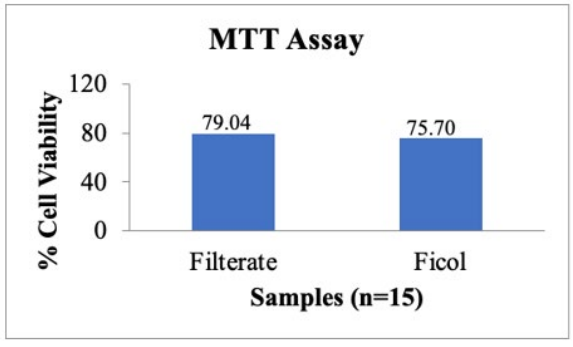

Figure 3

Insulin concentration plays a very important role to prove the functionality of the human pancreatic islet cells. We analyzed the insulin concentration by chemiluminescence assay in the purified islet samples and figure 4 shows the concentration of all samples against the standard values.

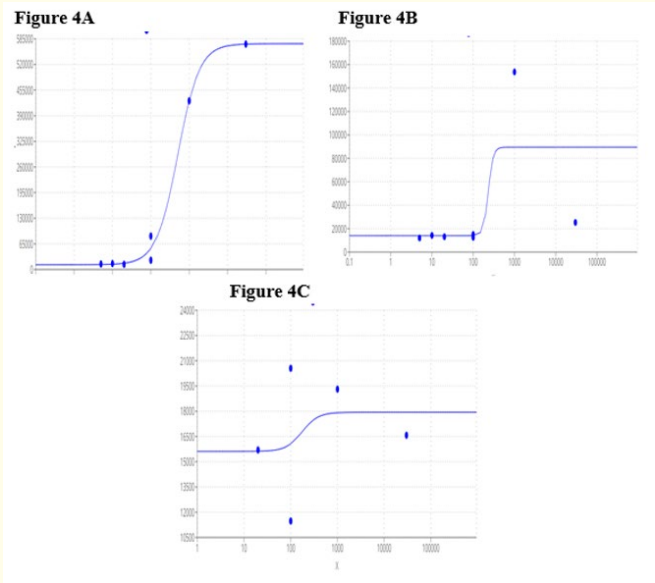

Figure 4: Figure 4A represents concentration of standards vs RLU units of standards. Figure 4B represents concentration of standards vs RLU units of unknown samples. Figure 4C represents concentration of standards vs RLU units of unknown samples.

Discussion

This research work comprehensively analyses the various facets of 15 islet isolations exclusively from an Indian population. Our short experience in the Indian population of isolating human islets suggests that many factors affect the ability to recover human islets. This paper evaluates 15 human islet isolations along with clinical variables and its purification process by two varied methods which were assessed for viability and functionality.

Islet transplant programs and distribution of human islets is less facilitated in Asian countries like India owing to a lack in networking and coordinating efforts. Significant reasons for this might include a deficit in pancreas donors, cultural and legislative barriers leading to a shortage in proper organ procurement

programs, and a mentionable resource insufficiency in establishing and maintaining human islet isolation infrastructures [23]. Islet transplantation can be advantageous if translated to a clinical reality owing to its less-invasive procedure, low morbidity and technical ease in carrying out repeated transplantation of islets compared to pancreas transplantation. Though advantageous over pancreas transplantation, islet transplantation (both allo- and auto-transplantation) still remains an experimental process in India. This study can thus bring an optimistic change for countries that are yet to initiate the experimental procedures and establish the islet transplant program.

In India, Islet transplantation should be considered as the therapeutic approach for type 1 diabetes. Hence an efficient, economic, and rapid isolation of islets is necessary for effective therapy. Over the past few decades, the isolation and purification of islets were carried out by tissue mincing and digestion, followed by mechanical grinding and digestion; however, both the yield and quality of the resulting islets needs to be improved [24-26].

There are various factors which can affect the quality of islet preparations, and such factors are clearly stated in Table 1 and Table 2 of our research findings. In accordance with other studies, we also observed donor characteristics to be a major factor influencing islet preparations' quality [27-30]. In these fifteen samples, age had a negative correlation with islet yield. Some studies observed a better yield of IEQs from donors having higher BMI [31,32]. Lyon., *et al*. correlated higher donor BMI with larger islet size but with either islet yield, purity or its function [27]. Our study has inferred BMI to correlate positively with the islet yield. According to Wrenshall., *et al*. pancreatic insulin content was positively correlated with increased body surface area [33]. Islet yield gets impacted by cold ischemia time (CIT) of the tissue [Table 3]. The CIT is the time period commencing from the time of procurement and preservation of the organ until it's processed at the islet isolation facility [34]. A CIT of below 12 hours demarcated for clinical islet transplantation can pose a barrier for sample count by minimizing the acceptance rate, especially in areas where the nearest isolation facility is not in proximity [35,36]. Extending the pancreas CIT by up to 24 h moderately impacts the islet yield [27]. And this was also evident in our finding where the islet yield correlated negatively with the CIT; in spite of excluding a few samples which had exceptionally long CIT due to logistic reasons. Nevertheless, for carrying out basic research on islet isolation, donor pancreas with an extended CIT can still be an option.

A recent study demonstrates the feasibility of clinical islet transplantation; however, most recipients require several islet infusions [37]. The reasons underlying this requirement for islet redosing are likely to include islet loss caused by both immunologic and nonimmunologic mechanisms. Even in a well-established center, there is considerable variability in islet yield per donor and in the number of islets required to ensure effective islet mass. In this regard, exposure to collagenase, endotoxin, and Ficol during islet isolation may cause release of inflammatory mediators *in vitro* [38-40]. These could contribute to inflammation, apoptosis, and immunologic attack *in vivo*. Therefore, changes in the isolation procedure that increase efficiency while reducing injury should augment functional islet mass, decrease primary nonfunction, and improve long-term engraftment. Here, we describe a simple purification procedure that confers these advantages by filtration of digested pancreata through a cell strainer, thereby avoiding Ficol density centrifugation and its associated toxicity. Our study signified that, both the methods (that is, 'ficol density centrifugation' and 'purification by filtration'), ensured that the islets were intact and separable from the non-digested tissue. Purity was also comparable between the methods [Figure 3]. Noteworthy was a finding that, 'purification by filtration' was relatively better in speed, viability, islet yield and had an outcome of higher in-vitro insulin secretion. To our knowledge, this is the first experimental study in the Indian population which demonstrates that exposure to ficol during isolation translates into decreased islet viability or function when compared to purification by filtrate method.

Islet function is defined by its ability to regulate the release of insulin and other hormones in response to changes in extracellular glucose concentration [41]. Interestingly, the insulin release from the human pancreatic beta cells is well illustrated in figures 4A, 4B, 4C and 5. Different samples based on their varied clinical conditions, released insulin in the range of 4 to 250pg/ml. Islet size is also an influencing factor of its functionality. Some of the research papers have noted the superiority of rodent and human islets under 100-125 µm against large islets in *in vitro* and *in vivo* function [42-44]. In this regard, Fujita., *et al*. [45] observed that larger the islet size, lesser becomes the insulin secretion per islet

equivalent. Consistent to this fact, in our human pancreata, small islets hold a higher proportion of beta cells compared to the larger ones. Adding on to this, the proximity of small islets with blood vessels also impacts the insulin secretion [42]. However, in a more recent study by Nam and colleagues [44] the islets with the highest SI value had between 100 and 150 µm diameter in a heterogeneous population. Additionally, we are yet to study if 100 µm filtration could lead to loss of some islets during the filtration process. But as per Miriam., *et al*. 2015, has stated that there was no statistical differences in Stimulation Index value with islets over 100 µm (2.12 ± 0.22 vs. 1.77 ± 0.20 for islets under 100 µm and over 100 µm by filtration, p = 0.2615 [45].

Taken together, our results show that the quality of the islets obtained by both methods is good, while the speed of filtration using a 100 µm cell is particularly convenient for large scale islet isolation and/or laboratories with limited staff. Besides, we made a balance considering also the cost and the time consumed by each procedure, which are also determinant in the election of a method for a laboratory routine. It is currently accepted that mouse and human islets differ in their anatomy and functionality [46] and that the human pancreas is more fibrous and dense than in rodents [47,48]. But both human and rodent islets are in the same range of size [49], thus making size-based purification like filtration a method of interest also for human islet purification. We conclude that filtration is a feasible alternative to ficol, providing good quality islets, and very convenient in terms of time and cost for large-scale islet isolations and/or laboratories with limited staff. However, each laboratory should decide its most suitable option.

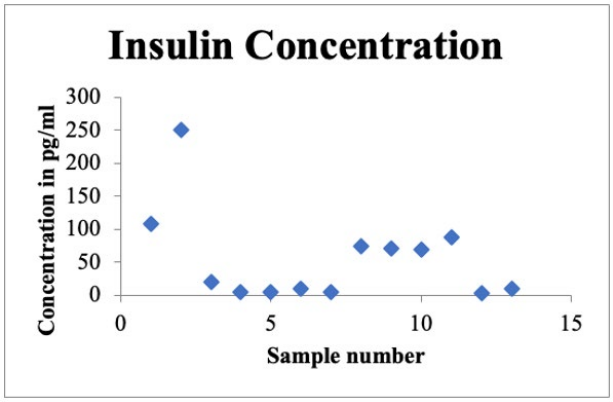

Figure 5: Explains the Insulin concentration of unknown samples ranges from 4-250 pg/ml.

Conflict of Interest

We hereby declare that we have no conflict of interest of any form pertaining to our research study titled, "A Simple, Time Efficient Purification Methodology Providing Good Quality Human Pancreatic Islets In The Indian Population".

Funding Support

We would sincerely thank the Indian Council for Medical Research (ICMR) [ICMR-5/3/8/76/2020-ITR, 24-12-2020] for the encouragement and support extended to bring this process technology to India. Special Permission was given to use the pancreatic tissue for research after the ethical approval.

Acknowledgements

- Indian Council for Medical Research (ICMR), New Delhi
- Apollo group of Hospitals, Chennai
- Apollo Hospital and Research Foundation, Chennai
- Dr. Balamurugan Appakalai, USA
- Velammal Hospital and Medical College, Madurai
- Biotechnology Industry Research Assistance Council (BIRAC)
- IKP Knowledge Park, Telangana
- MS Swaminathan BioIncubation Centre
- Crescent Innovation and Incubation Council.

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