Calcium: A Crucial Potentiator for Efficient Enzyme Digestion of the Human Pancreas

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Abstract

Background: Effective digestive enzymes are crucial for successful islet isolation. Supplemental proteases are essential because they synergize with collagenase for effective pancreatic digestion. The activity of these enzymes is critically dependent on the presence of Ca^{2+} ions at a concentration of 5–10 mM. The present study aimed to determine the Ca^{2+} concentration during human islet isolation and to ascertain whether the addition of supplementary Ca^{2+} is required to maintain an optimal Ca^{2+} concentration during the various phases of the islet isolation process. **Methods:** Human islets were isolated according to standard methods and isolation parameters. Islet quality control and the number of isolations fulfilling standard transplantation criteria were evaluated. Ca^{2+} was determined by using standard clinical chemistry routines. Islet isolation was performed with or without addition of supplementary Ca^{2+} to reach a Ca^{2+} of 5 mM. **Results:** Ca^{2+} concentration was markedly reduced in bicarbonate-based buffers, especially if additional bicarbonate was used to adjust the pH as recommended by the Clinical Islet Transplantation Consortium. A major reduction in Ca^{2+} concentration was also observed during pancreatic enzyme perfusion, digestion, and harvest. Additional Ca^{2+} supplementation of media used for dissolving the enzymes and during digestion, perfusion, and harvest was necessary in order to obtain the concentration recommended for optimal enzyme activity and efficient liberation of a large number of islets from the human pancreas. **Conclusions:** Ca^{2+} is to a large extent consumed during clinical islet isolation, and in the absence of supplementation, the concentration fell below that recommended for optimal enzyme activity. Ca^{2+} supplementation of the media used during human pancreas digestion is necessary to maintain the concentration recommended for optimal enzyme activity. Addition of Ca^{2+} to the enzyme blend has been implemented in the standard isolation protocols in the Nordic Network for Clinical Islet Transplantation.

Keywords

calcium, clinical islet transplantation, diabetes, islet isolation

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Introduction

Clinical islet transplantation constitutes an efficient treatment option for subjects with severe type 1 diabetes complicated by a lack of hypoglycemia awareness. Compared with whole-pancreas transplantation, islet transplantation has a low frequency of serious complications[1]; an outstanding problem, however, is inconsistency in the ability of particular enzyme blends to catalyze efficient digestion of the pancreas, allowing liberation of a large number of islets with intact function. This problem has stimulated huge efforts toward characterizing and standardizing enzymes for islet isolation, and currently several manufacturers provide GMP-grade (Good Manufacturing Practice) products. Even so, each islet isolation center experiences not only lot-to-lot variation in digestion effectiveness but also a significant variability between individual pancreata. Surprisingly, an enzyme deemed insufficient in one islet isolation facility can work properly when evaluated in another center. These experiences point to a thus-far unrecognized critical component governing enzyme efficiency during clinical islet isolation that cannot be detected by standard assays of enzyme activity. For decades, Ca^{2+} has been known to be essential for the enzymatic activity of collagenase, neutral protease, thermolysin, and clostripain[2–5], and all manufacturers who make these enzymes available add Ca^{2+} to their enzymes. However, the total amount of Ca^{2+} provided is not sufficient when preparations are further diluted to 200–400 ml, as is usually done for clinical islet isolation. Therefore, the Clinical Islet Transplantation (CIT) protocol stipulates Ca^{2+} supplementation of the digestion solution with a target concentration of 5 mM for collagenase from two manufacturers (Roche and Serva)[6].

The aim of the present paper was to examine the actual Ca^{2+} concentrations in the medium during the various phases of clinical islet isolation and to determine whether the further addition of Ca^{2+} is required during the islet isolation process to obtain the recommended concentration.

Materials and Methods

Organ Procurement

All participating centers within the Nordic Network for Clinical Islet Transplantation (NNCIT; http://nordicislets.medscinet.com) used standard organ procurement procedures[7]. Centers within the NNCIT include the University Hospital, Gothenburg; University Hospital, Malmö; Karolinska Institute, Stockholm; Uppsala University Hospital, Uppsala; the Surgical Hospital, Helsinki University, Helsinki; Oslo University Hospital, Rikshospitalet, Oslo; and Rigshospitalet, Copenhagen.

Donor, Transport, Islet Isolation, and Islet Maintenance

Donors were selected by applying the same criteria as for clinical kidney donation in the Scandiatransplant region, but pancreata were only retrieved from donors of 25–70 years of age. Exclusion criteria included a glycosylated hemoglobin A1c (HbA1c) >48 mmol/mol according to the International Federation of Clinical Chemistry and Laboratory Medicine[8].

Statistical analysis of the donor characteristics and parameters of the islet isolation was performed using a nonparametric test.

Details for islet isolation were described previously[9,10]. All standard operating procedures are available at http://nordicislets.medscinet.com. A selected enzyme blend consisting of collagenase, thermolysin (both Roche, Indianapolis, IN, USA), and clostripain (Vitacyte, Indianapolis, IN, USA) was utilized.

Samples taken from the dissolved enzyme blend during the recirculation and harvest phases were analyzed on an Abbott Architect c16000 platform (Department of Clinical Chemistry, Uppsala University Hospital, Uppsala, Sweden)[11]. The $[Ca^{2+}]$ in all solutions used during the isolation process was analyzed directly after preparation and at various time-points during the islet isolation process, as indicated in Figure 1.

In total, 19 isolations were performed between January and November 2017. The isolations in the control group ($n = 9$) were performed according to the NNCIT's standard procedure. In the group with supplementary Ca^{2+}, one isolation was performed following the protocol of the National Institutes of Health Clinical Islet Transplantation Consortium (CIT) using 10 mM of Ca^{2+} in HBSS for dissolving the enzymes (see Table 1), followed by ordinary CMRL culture medium in the recirculation and harvest phases of the isolation. Since the CIT protocol is not our standard protocol and we were only interested in $[Ca^{2+}]$ during the isolation process, we have not included this isolation in the evaluation.

The subsequent nine isolations that included Ca^{2+} supplementation were performed using Ringer's acetate-based solutions according to the NNCIT's standard procedure. Initially the $[Ca^{2+}]$ was adjusted to 5 mM only in the stock solution used for dissolving the enzymes (Table 1). A second isolation was performed by increasing the $[Ca^{2+}]$ to 5 mM in the stock solution as well as during the harvest phase. During a third isolation, both the stock solution and the recirculation solution had a $[Ca^{2+}]$ of 5 mM, and the $[Ca^{2+}]$ in the medium for harvest was 4 mM. The lessons learned from these first isolations were implemented to yield a $[Ca^{2+}]$ of 5–7 mM during the entire islet isolation process performed on an additional six consecutive pancreata.

Islet Quality Control

All functional and viability analyses were performed according to standard protocols (http://nordicislets.medscinet.com/en.aspx). The day after islet isolation, quality analyses were performed using a dynamic glucose-stimulated insulin secretion (GSIS) perfusion test (Brandel, London, UK) (Figure 2) on 20 handpicked islets to calculate the stimulation index
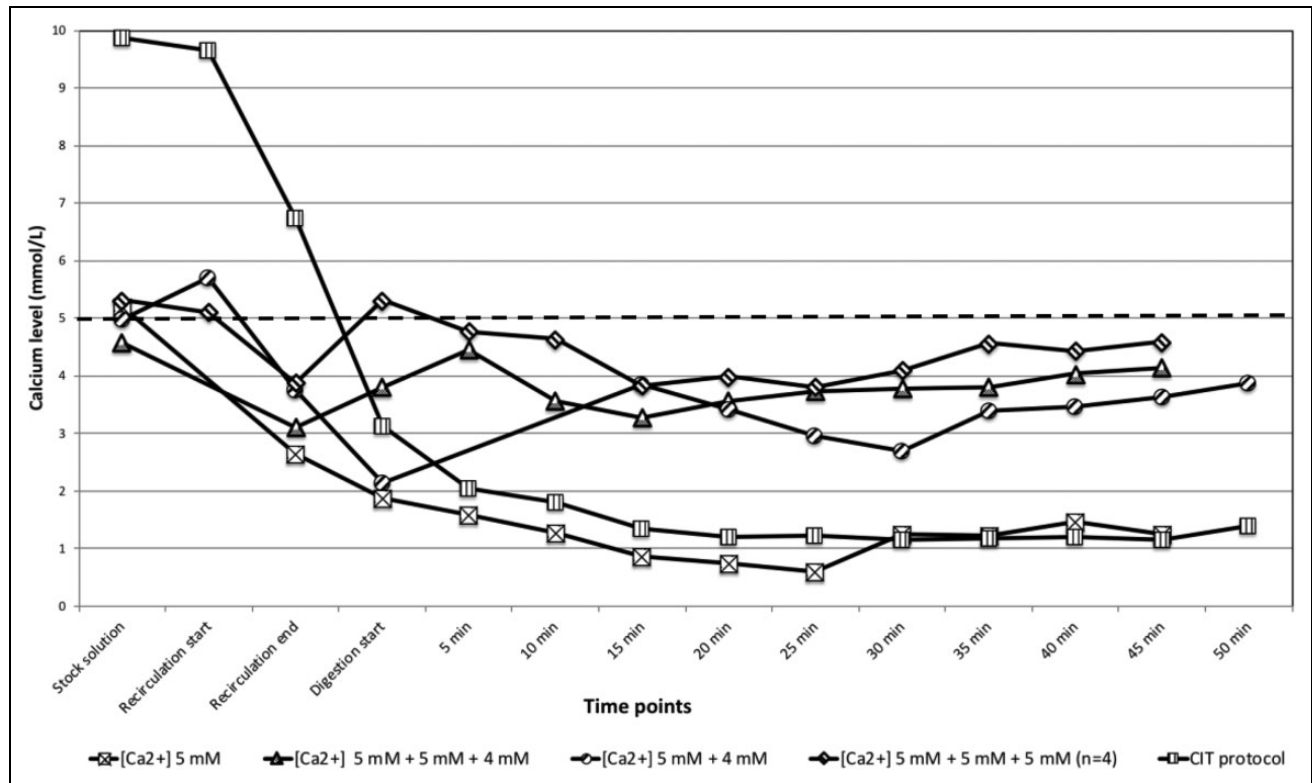
Figure 1. Calcium levels measured during the islet isolation process. The dotted line indicates a calcium level of 5 mmol/L.

Table 1. Addition of Supplementary Calcium to Media to Achieve the Target Concentrations Indicated.

Islet isolation	1	2	3	4–7	8–9	10 (CIT protocol)
Enzyme stock solution	5 mM	5 mM	5 mM	5 mM	7 mM	10 mM
Recirculation	–	5 mM	–	5 mM	7 mM	–
Harvest	–	4 mM	4 mM	5 mM	7 mM	–

(average of the high-glucose phase divided by the average of the low-glucose phase)[12]. Islet quality control in one isolation was performed using a modified protocol that mainly included a shortened perfusion period. All insulin values were measured using a human insulin-specific ELISA (Mercordia, Uppsala, Sweden).

CUSUM Test

The cumulative sum chart test (CUSUM) is a sequential analysis technique used for monitoring change in relation to a set number – for example, the mean number of islet equivalents (IEQ) isolated per pancreas. For each isolation, the difference in IEQ, negative or positive, in relation to the mean is calculated. Whenever there is a systematic increase or decrease in the number of IEQ obtained, a deviation of the curve from the mean occurs.

Results

Donor Characteristics

The donor characteristics for the islet isolations involving supplementary Ca^{2+} ($n = 9$) and a control group ($n = 9$) following the NNCIT protocol were not significantly different regarding donor age, gender, body mass index (BMI), HbA1c, cold ischemia time, dissection time, trimmed pancreas weight, digestion and harvest time, pellet volume before separation, and amount of pancreas left in the chamber. Neither were the parameters for the donor from the one isolation following the CIT protocol.

Ca^{2+} Concentration During the Islet Isolation Process

HBSS is used as the basic fluid during the isolation process in the CIT network, while Ringer's acetate is used in the Nordic network, but both contain 2 mM Ca^{2+} without additives. When we followed the CIT protocol for supplementation of Ca^{2+}, [Ca^{2+}] levels reached the expected 10 mM level in the stock solution but had already dropped dramatically during the recirculation phase (Figure 1).

Similarly, the addition of Ca^{2+} increased the [Ca^{2+}] to 5 mM in Ringer's acetate. Adding Ca^{2+} only to the stock solution raised the [Ca^{2+}] to 5 mM, but it dropped to 2 mM during the recirculation phase and to <1 mM during the collection phase. When we added additional Ca^{2+} to the
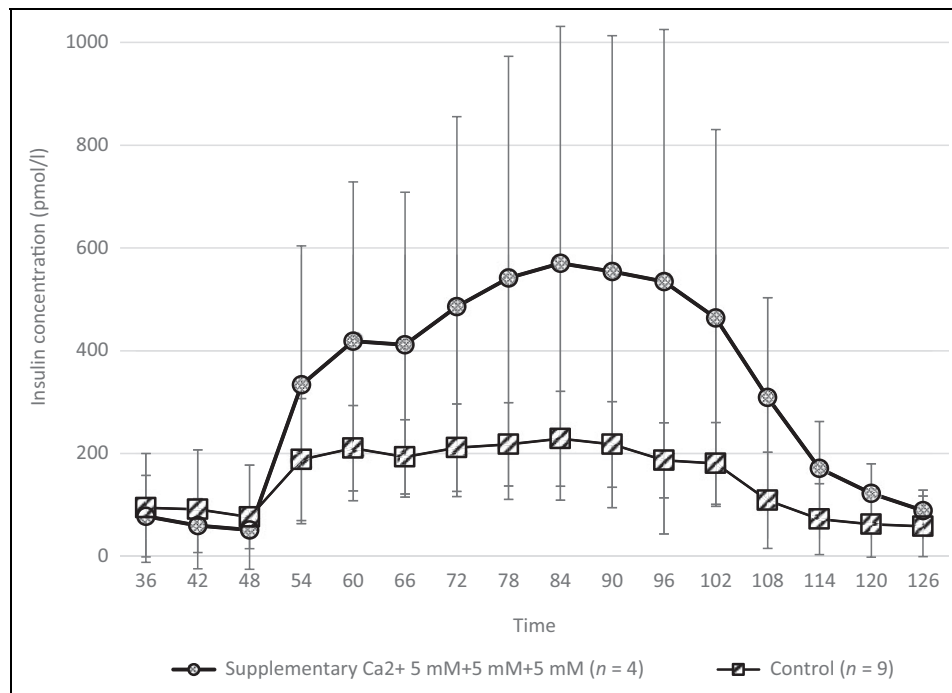
Figure 2. Insulin release curve in response to dynamic glucose perfusion. Islets were perfused with high glucose (16.7 mM) from 42 to 84 min, whereas other fractions were exposed to low glucose (1.67 mM). Data shown from the final series of isolations using supplementary Ca^{2+} of 5 mM in all solutions.

stock solution and to the fluids used during the collection phase, the expected rise in $[Ca^{2+}]$ to 5 mM was confirmed, followed by a continuous drop to <3 mM during the recirculation and digestion phases and a slight increase again when we used Ringer's acetate supplemented with Ca^{2+} to target a concentration of 5 mM. When we adjusted the $[Ca^{2+}]$ levels to 5 mM in the stock solution and recirculation phase and also to 4 mM during the collection phase, we saw only a minor dip during the recirculation phase, and the levels remained stable during the collection phase. The $[Ca^{2+}]$ was ~ 5 mM during all three phases when the level was adjusted to 5–7 mM in all solutions (Figure 1).

Isolation Results

The results of the isolations in the supplementary Ca^{2+} group and the control group were significantly different only for the number of IEQ, which was significantly higher ($P < 0.05$) for the supplementary Ca^{2+} group: 323,730 (range 202,500–453,739) vs. 147,319 (range 55,978–266,086); there was no significant difference ($P > 0.05$) between the groups regarding the purified tissue volume, IEQ/g pancreas, total purity, and recovery after 1 day of storage (Table 2). Islet size distribution was comparable to that previously published (Figure 3)[13].

The quality assessment of the islets revealed no negative impact of the additional Ca^{2+} during the various periods on the SI values (3.0 [range 1.3–5.5]) or levels of insulin release. The GSIS perfusion curves showed stimulated

insulin release, with an initial spike followed by continued and sustained insulin release until a return to low glucose levels. Data are shown only for the isolations with supplementary Ca^{2+} during the entire isolation process (Figure 2).

The cumulative plot of the IEQ for islet isolation according to the NNCIT protocol showed an increasing number of cumulative IEQ when additional Ca^{2+} was used to obtain a $[Ca^{2+}]$ of 5–7 mM in all media, when compared to a series of consecutive isolations using the standard procedure with extra Ca^{2+} supplementation only in the stock solution used for dissolving the enzyme blend (Figure 4).

Discussion

The results presented here indicate that the concentration of free Ca^{2+} during the islet isolation procedure, conducted according to the present CIT recommendations and other standardized protocols for clinical islet isolation, often can be below 1 mM even under conditions in which supplementary Ca^{2+} has been added to achieve an initial target concentration of 5 mM. It is well known that when Ca^{2+} is added to bicarbonate-buffered solutions, precipitation of calcium carbonate will occur, thereby reducing the amount of free Ca^{2+}; however, this reaction has thus far been overlooked in the field of clinical islet isolation.

Calcium carbonate ($CaCO_3$) is a common substance that occurs in rock in the form of the minerals calcite and aragonite (found, for example, in limestone). It is the main component of pearls and the shells of marine organisms, snails,

Table 2. Characteristics of Donors and Donor Pancreata.

	Supplementary Ca^{2+} ($n = 9$)		Control ($n = 9$)			
	Mean	Range	Mean	Range	*P* value ($P < 0.05$)	
Donor age	55 years	34–69	61 years	54–70	0.477	ns
Gender (male/female)	6/3	NA	4/5	NA	NA	
BMI	28.2	23.9–33.1	25.5	21.6–30.5	0.7414	ns
HbA1c	37.7	31.0–47.0	38.2	34–41	0.28914	ns
Cold ischemia time	09:59 h	04:41–16:55 h	10:22 h	03:48–21:28 h	0.85716	ns
Dissection time	0:54 h	0:38–1:14 h	0:52 h	0:44–1:11 h	0.92828	ns
Trimmed pancreas	140 g	80–292 g	93 g	41–144 g	0.151	ns
Digestion time	16 min	8–24 min	24 min	16–57 min	0.6432	ns
Harvest time	0:37 h	0:27–0:52 h	00:45 h	00:31–1:11 h	0.18684	ns
Pellet volume before separation	45 ml	20–85 ml	33 ml	10–51 ml	0.7672	ns
Pancreas left in the chamber	22 g	5–61 g	19 g	7–50 g	0.89656	ns
Extent of pancreas digestion (%)	85.3	57.6–96.2	80.8	66.2–93.1	0.4009	ns
IEQ	323,730	202,500–453,739	147,319	55,978–266,086	0.0027	$P < 0.05$
Purified tissue volume (μl)	2196	710–5990	1125	350–1,525	0.05744	ns
Total purity (%)	44	14 -71	37.7	12–62	0.45326	ns
IEQ/g pancreas	2852	1867–3,969	2296	749–5,676	0.13362	ns
Recovery after 1 day (%)	109	82–136	106	58–145	0.05118	ns

BMI, body mass index; IEQ, islets equivalent.
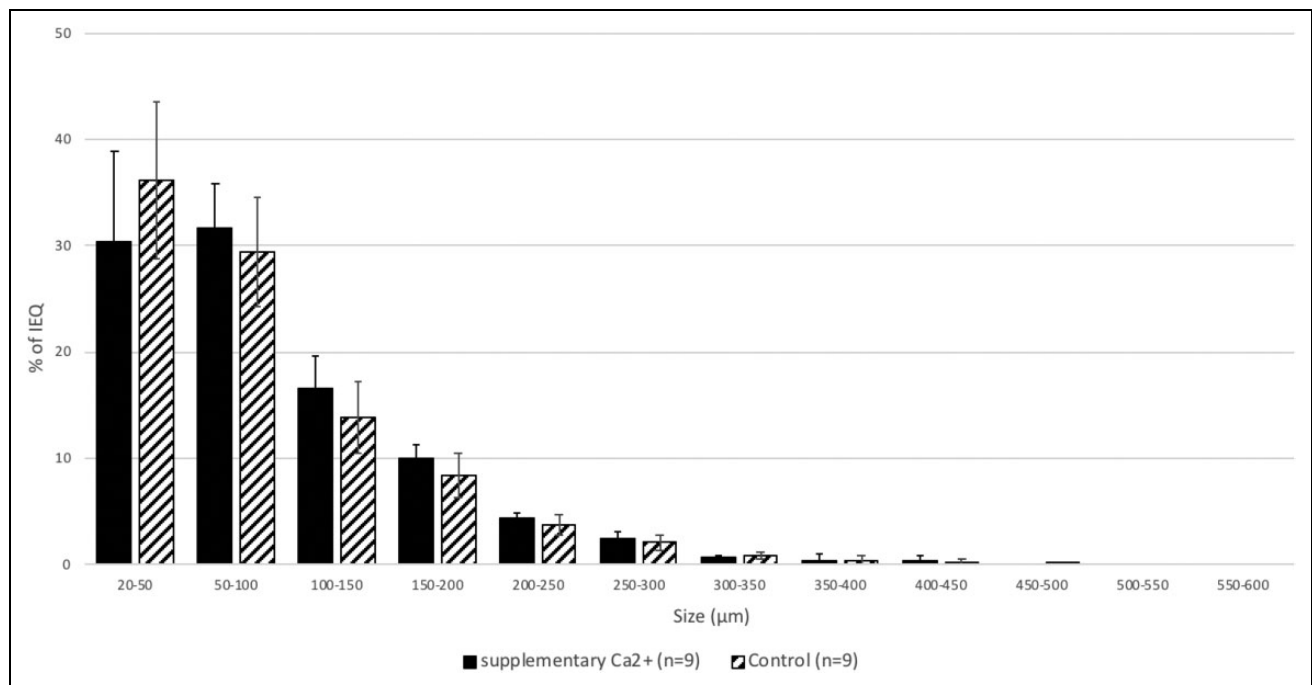
Figure 3. Islet size distribution as a percentage of total IEQ number. Data from one isolation following the CIT protocol are not included.

and eggs. Calcium carbonate is created when calcium ions react with carbonate ions. Once formed, calcium carbonate is an insoluble precipitate that will not dissolve even if diluted in large volumes of fluid. Based on the results presented here, the use of bicarbonate-buffered solutions such as HBSS and of bicarbonate for pH adjustment, both recommended by the CIT, should be avoided. Several other buffer systems can be used to avoid the formation of calcium carbonate, such as

a HEPES-based buffer, together with pH adjustment using sodium hydroxide.

More surprising was our finding of a significant consumption of Ca^{2+} during the islet isolation procedure. The mechanism(s) through which Ca^{2+} is consumed is/are currently unknown. However, during digestion, Ca^{2+} is exposed to the extracellular matrix (ECM) of the pancreas. The ECM is composed of various highly negatively charged
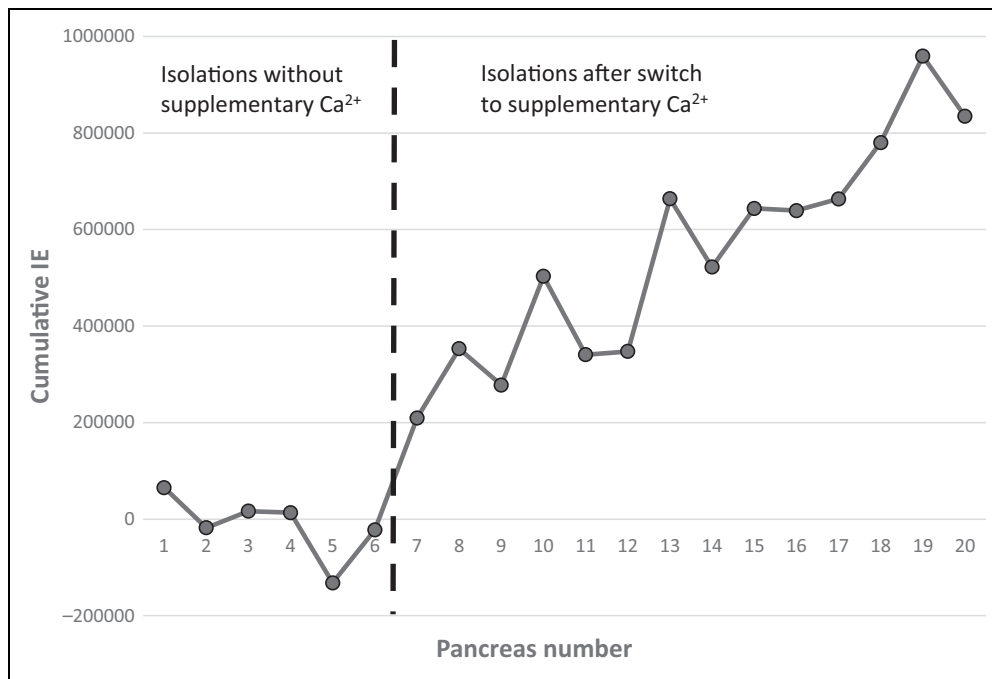
Figure 4. Cumulative sum chart test (CUSUM): cumulative plot of islet equivalent (IEQ) obtained after successive pancreas islet isolations following the NNCIT protocol before and after the implementation of supplementary Ca^{2+} during the entire islet isolation process. The dotted line indicates the switch. For every islet isolation, the difference from 200,000 IEQ is plotted. If the number of IEQ is above 200,000 IEQ, the next plot point is a positive addition to the graph; if below, a negative addition. The dotted line indicates the switch to Ca^{2+} supplementation as part of the islet isolation process.

macromolecules[14], such as laminins, collagens, aggrecan, heparan sulfate/heparan sulfate proteoglycans, and hyaluronan, which are liberated in large amounts during digestion and can potentially bind large quantities of Ca^{2+}[15].

Irrespective of the cause, we found that the $[Ca^{2+}]$ during isolation fell to concentrations that are known to negatively affect enzyme activity[2–5]. To counteract this loss of free Ca^{2+}, the media used during enzyme perfusion of the pancreas, digestion, and harvest must be supplemented with Ca^{2+}.

A validated digital image analysis technique was used in the present study to avoid bias, investigator variations, and overestimation of the islet number and purity[16,17]. The quality of the islets was good, with no observable negative effects of the procedure on the islet number, purity of the islets, size distribution, or islet recovery after culture. Similarly, the dynamic GSIS showed well-preserved islet function, and all islet preparations were considered acceptable for clinical use.

Supplementation with Ca^{2+} seems to improve the efficacy of the enzyme blend without causing any negative effect on islet number, purity, or function. The rate of clinical islet transplantations is envisioned to increase substantially if a Ca^{2+} concentration optimal for enzyme activity is applied. From a health care and insurance perspective, the increased rate of transplantation itself should translate to a substantial improvement in the cost–benefit of islet

transplantation[18]. Furthermore, potentiation of the enzyme blend by Ca^{2+} supplementation may allow the use of lower quantities of enzymes during human islet isolation, further reducing the cost of clinical islet isolation.

The relative importance of Ca^{2+} in the digestion of a specific pancreas may vary between donors – that is, digestion of the pancreas from some donors may be almost complete without supplementation with Ca^{2+}, whereas in other cases the addition of Ca^{2+} may be essential for efficient digestion. The currently used enzyme blend was of good efficiency, but as usually observed, there was variation in the digestion efficiency between donors. Notably, this blend of enzymes showed almost no digestion of a pancreas retrieved from one optimal organ donor. Macroscopically, the pancreas was without surrounding fat, and examination after dividing the pancreas into pieces before digestion demonstrated an organ without fibrosis or infiltrating fat. The center in Uppsala currently has experience with more than 2300 human pancreata, and this pancreas was deemed to be among the top 20 from a quality perspective. However, after digestion for 25 min, the islet isolation was stopped because no material was released from the Ricordi chamber. Examination revealed perfectly intact pieces of pancreas without any sign of digestion. Based on this experience, the present study was initiated. We speculated that the variability in digestion efficiency between pancreata might be caused by variable release of Ca^{2+} from intracellular storage

– for example, that liberated from lysed adipocytes. This notion is in agreement with the frequently reported beneficial islet isolation outcomes obtained from pancreata with an ischemic time of 2–4 h, as compared to those processed immediately after procurement[19].

In summary, additional supplementation of the media used in clinical islet isolation with Ca^{2+} beyond the 5 mM traditionally in stock solutions is required to achieve the recommended $[Ca^{2+}]$ of 5–7 mM to obtain optimal enzyme efficiency. Ca^{2+} supplementation improves the digestion efficacy and kinetics for consistent release of a high number of intact and fully functional islets. Since no detrimental effects on islet function or viability were observed, Ca^{2+} supplementation, as described here, is now in standard use at the islet isolation centers within the NNCIT, showing a positive trend in islet isolation outcome (Figure 4).

Ethical Approval

The study was approved by the Regional Ethics Committee in Uppsala (Dnr 2009/043, 2009/371) and Stockholm (Dnr 2017/1471-32), Sweden (http://www.epn.se) according to the Act concerning the Ethical Review of Research Involving Humans (2003:460).

Statement of Human and Animal Rights

This article does not contain any studies with human or animal subjects.

Statement of Informed Consent

All work involving human tissue was conducted according to the principles expressed in the Declaration of Helsinki and in the European Council's Convention on Human Rights and Biomedicine. Consent for organ donation (for clinical transplantation and for use in research) was obtained from the relatives of the deceased donors by the donor's physicians and documented in the medical records of the deceased subject.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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