A New Method for Quantification of Islets by Measurement of Zinc Content

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The ability to quantify the yield of pancreatic islet tissue after isolation is important for interlaboratory comparisons and for the assessment of islet yield prior to clinical transplantation. Because pancreatic islets contain a much higher concentration of zinc than other tissues, we investigated the analysis of zinc as a measure of islet tissue yield. Rat islets of standard diameter 250 μm were handpicked into samples containing 10–80 islets. The zinc content was measured by EAAS and showed a linear correlation with islet number. A zinc binding fluorescent dye, TSQ, was investigated as a way of simplifying the zinc measurement for routine use. Samples of 10–80 islets of 250 μm were sonicated in 3 ml zinc-free water, 0.18 μmol TSQ was added, and the TSQ-zinc fluorescence was measured at 480 nm. A linear correlation was observed. Exocrine contamination up to 50% barely affected the results. Islet zinc content also was shown to be correlated linearly with islet number for freshly isolated human islets. Measurement of zinc by TSQ fluorescence is a rapid, cheap, and objective measure of islet tissue content. *Diabetes* 41:1056–62, 1992

A number of centers are engaged in clinical trials of human islet transplantation, in which the quality of the islet preparation is probably the single most important variable affecting islet transplant results (1). The number of islets obtained from a single pancreas is dependent upon many factors, including the state of the donor pancreas (2), the preservation solution used (3,4), the agents used for collagenase digestion (5,6), and the media used for density gradient purification (7,8). Progress has been made in each aspect of the isolation procedure, culminating recently in successful clinical islet transplants (9,10). To allow further development of islet isolation techniques, it is important that interlaboratory comparisons and assessment of yields prior to clinical transplantation can be made. To this end, a simple, reliable technique for quantifying islet yields is needed. Counting islets is not the optimal method, as islets vary in size from <50 μm to >500 μm in diameter (11). Because the pancreatic yield can vary from 365,000–590,000 islets from a single human pancreas (10), a sampling method to quantify the islets is necessary. Initially, the islet yields were expressed as islets per gram of donor pancreatic tissue (12,13), however, the errors caused by variation in the size of islets are enormous. An improved technique has been to size each islet in the sample and calculate the total endocrine volume for the sample using the volume formula for a sphere (14). The total islet volume then is converted to 150 μm islet equivalents. Recent reports of clinical human islet trials have used the 150 μm islet equivalent method (9,10). However, the sizing of islets is performed by eye, placing the islets within size bands. In addition, the formula used to calculate volume assumes a spherical shape, which is certainly not uniformly true for islets, thus errors are still likely to be considerable.

Gagerman (15) considered the measurement of islet protein as a measure of islet mass for islet quantification in biochemical studies, but this approach is applicable only to fully purified islets because protein content of exocrine tissue is similar to that of islets. It is known that islets contain large amounts of zinc compared with exocrine tissue (16,17), and we have investigated the measurement of zinc content as an estimate of islet yield. To measure zinc content accurately, we used EAAS, an extremely sensitive method for measuring trace elements

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Received for publication 8 August 1991 and accepted in revised form 20 March 1992.

EAAS, electrothermal atomization atomic absorption spectrophotometry; TSQ, N-(6-methoxy-8-quinolyl) paratoluenesulfonamide; BSA, bovine serum albumin; RIA, radioimmunoassay.

(18,19,20). EAAS showed a linear correlation with islet yield and islet protein, and a good correlation with islet insulin. Because EAAS is a complex and expensive method of measuring zinc, we subsequently developed a technique using a zinc-binding fluorescent dye, TSQ, to measure the zinc/TSQ fluorescence by a commonly available fluorimeter. This result was a new, objective—and cheap—method for quantifying islet yields.

RESEARCH DESIGN AND METHODS

PVG ($RT1^c$) rats (Olac, Blackthorn Farms, Bicester, Oxon, UK) were used for all the experiments with rat islets. Human islets were isolated from pancreases obtained from cadaveric donors, with relevant permission given by relatives.

Rat islet isolation. The method of Sutton et al. (21) was used for rat islet isolation. Briefly, the rats were exsanguinated under anesthesia, the bile duct cannulated, and Hanks solution (Flow Labs, Irvine, Scotland) containing 7.5 mM calcium chloride, 20 mM HEPES buffer, and collagenase (1 mg/ml of type 11, Sigma, St. Louis, MO) was injected to uniformly distend the pancreas. The pancreas then was incubated for 20 min in Hanks solution at 39°C, followed by transfer into Hanks solution containing 5 g/L BSA and 20 mM HEPES buffer at 4°C. The pancreas was dispersed by gentle shaking and washed twice. The dispersed tissue was filtered through a nylon mesh of 500 μm pore size, the pellet of the filtered tissue was suspended in the bottom of a discontinuous gradient of two layers of BSA (Advanced Protein Products, Brockmoor, WestMidlands, UK) of 1.069 g/L and 1.053 g/L, and then overlaid with Hanks solution. After centrifugation at 1500 *g* for 15 min at 4°C, the tissue obtained from the interface of the two BSA layers was washed twice in normal saline prepared with purified water.

All subsequent steps were carried out in glass or plasticware, soaked for 24 h in 6 M nitric acid, and rinsed in purified water or 0.145 M sodium chloride (normal saline). Water was purified by a 5 μm cartridge filter followed by a SERAL mixed bed deioniser (BDH, Dagenham, Essex, England). Conductivity was <0.2 μS/cm. Analysis for zinc by atomic absorption gave no detectable signal. This water was used for the preparation of reagents for analytical procedures.

Human islet isolation. Human islets were isolated from cadaveric pancreases by a previously described method (22) with modifications (23). Briefly, the pancreatic duct was cannulated and distended by injecting 2 ml/gm of Hanks solution containing 7.5 mM calcium chloride, 20 mM HEPES buffer, and collagenase (3 mg/ml of type 11, Sigma). Biopsies of the gland were taken and incubated separately in dithizone solution (29). The pancreas then was incubated in Hanks solution at 39°C, until digestion was judged to be complete by examination of the biopsies, followed by transfer into Hanks solution containing 5 g/L BSA and 20 mM HEPES buffer at 4°C. The pancreas then was dispersed by a process of teasing with forceps and trituration; the tissue was washed further and passed through a 500 μm pore size mesh. The islets then were purified by centrifugation on a preformed BSA density gradient (densities 1.10/1.069/1.063/1.060/1.053). Islets of 250 μm diameter were hand-picked (as we did with the rat islets).

Method of handpicking islets. The washed islets were aspirated using an acid-washed glass pipette into a petri dish, and handpicked using a micropipette and a graticule eyepiece for sizing the islets. For the experiments below, all islets were size 10 on the graticule, corresponding to 250 μm in diameter.

TSQ stock solution. TSQ was obtained from Molecular Probes (Eugene, OR), and was used without further purification. Preliminary studies showed that it dissolved completely in DMSO at room temperature. TSQ stock solution was prepared at a concentration of 3.05 mM DMSO and stored at 4°C.

Method of measuring zinc by EAAS. Handpicked islets were transferred to weighed Eppendorf Safelock 1.5 ml plastic micro test tubes (Eppendorf, Germany). On receipt, these tubes were contaminated only slightly with zinc, which could be removed readily by acid washing. Regular checks on the washing procedure by analysis of water in these tubes showed no detectable zinc signal. All subsequent steps were carried out in these tubes to minimize contamination of islet samples with ambient zinc.

Samples of 10–80 islets were washed 3 times with 1.5 ml of 0.145 M sodium chloride (Aristar grade, BDH, Poole, Dorset, England) to remove residual zinc from the albumin gradient in which the islets were prepared. Islets were pelleted by centrifugation for 10 s at 50 *g* in an MSE Centaur centrifuge (MSE Instruments, Crawley, Sussex, England).

Water (350 μL) was added, and the samples were sonicated on ice at 50 W for 15 s with a DAWE Soniprobe Type 7532A (DAWE Instruments, England). A portion of the sonicate was removed for protein and insulin analysis. The volume of the remaining portion was determined by reweighing the tubes; and concentrated (15.7 M) nitric acid was added to give a final concentration of 1 M. The tubes were heated for 18 h at 90°C on a Tecam Dri-Block DB-3H heating block (Techne, Duxford, Cambridgeshire, England).

The acid digest was analyzed for zinc by sampling 5 μL directly from the Eppendorf tubes, which was analyzed by EAAS on a Perkin Elmer model 2380 spectrophotometer with HGA 400 graphite furnace and AS40 autosampler (Perkin-Elmer, Beaconsfield, Bucks, England). The sampling probe was flushed with 1.2 M Analar hydrochloric acid. The graphite tubes were coated pyrolytically. Standards in the range 0.05–1.0 μM in 1 M nitric acid were prepared from Sigma atomic absorption standard (Sigma, Poole, Dorset, England). The conditions for zinc analysis were largely those of Foote and Delves (24), except for data collection in the peak area mode and an integration time of 6 s. Peak heights were monitored on a chart recorder. Model 2380 settings were wavelength 213.9 nm, hollow cathode lamp current 15 mA, slit width 0.7 nm, deuterium background correction. The HGA 400 program is shown in Table 1.

TABLE 1
HGA 400 program*

Program step	Temperature (°C)	Ramp time (s)	Hold time (s)	Argon gas flow (ml/min)
1 (dry)	90	20	10	300
2 (dry)	110	10	10	300
3 (ash)	500	20	5	300
4 (ash)	500	1	4	60
5 (atomize)	2100	1	6	60
6 (clean)	2500	1	4	60

*It was established that these conditions were optimal for analysis of islet digests (data not shown).

Method of measuring tissue protein content. A portion of the tissue sonicate was analyzed for protein by a micro bicinchoninic acid method (25,26) (Pierce and Warriner, Chester, England). This method has similar sensitivity to the technique of Lowry et al. (27), but is technically simpler, having one working reagent rather than two. The chemistry is based on the biuret reaction. The reagent and colored product are less susceptible to interference from nonprotein substances. Sonicate (85 μL) and water (415 μL) were mixed with 500 μL protein assay reagent in 75 × 10 mm glass tubes. The capped tubes were incubated at 60°C for 60 min. Protein concentration was calculated from the absorbance at 562 nm using 0.05–20 mg/L BSA standards.

Measurement of fluorescence. All measurements of light absorption and emission were performed with a Perkin-Elmer LS 5 spectrometer on 3 mL samples. The cuvettes used were standard quartz, which were acid washed.

Statistics. Correlations were performed by least squares linear regression. Tests for significant differences between groups were carried out by Student's *t* test, with correction to allow for unequal sample variances where appropriate. The effect of exocrine contamination was analyzed by multiple regression analysis.

Insulin was assayed using a modification of the method of Albano et al. (28).

Experiments performed. The following seven experiments were conducted:

- Measurement of rat islet auto-fluorescence. Samples of 10–80 handpicked rat islets were sonicated in 3 ml saline. The auto-fluorescence was measured at 345 nm, excitation at 280 nm; and the peak wavelengths for auto-fluorescence were caused by protein content (15).
- Measurement of zinc content (fluorescence) of DMSO. As the TSQ was made up in DMSO, it was necessary to measure the background fluorescence attributable to DMSO alone. At excitation 365 nm and emission 480 nm, DMSO in the range 10–150 μL was added to samples of 10–80 sonicated islets.
- Measurement of zinc, protein, and insulin content of rat islets. Samples of 10–80 rat islets were washed to remove albumin, and were sonicated in water. Part of the sonicate was analyzed for protein by the bicinchoninic acid method and for insulin by RIA. A fraction of the sonicate was digested with 1 M nitric acid, and the zinc content measured by EAAS.
- Measurement of zinc and protein content of rat exocrine tissue. The exocrine tissue was separated from the pancreatic digest by staining with dithizone, which selectively stains islet tissue red, (29) allowing unstained exocrine tissue to be identified. The exocrine content was graded as 5–50% by spreading the tissue evenly on a petri dish and handpicking a percentage of the total area of the petri dish to give exocrine tissue for analysis in the range of 5–50%. The exocrine tissue was washed 3 times and sonicated. Protein was measured directly on 85 μL of the sonicate. Zinc was measured after the acid digestion step on 5 μL of the digest. To allow a valid comparison of the exocrine tissue zinc content with that of islets, zinc content was expressed as a ratio relative to protein content for each sample.
- Measurement of zinc/TSQ fluorescence of rat islets. Samples of 10–80 handpicked islets of uniform 250 μm diameter were sonicated, and the fluorescence measured by excitation at 365 nm and emission at 480 nm. The background fluorescence caused by 60 μL TSQ alone in zinc-free distilled water then was subtracted. The optimal concentration of TSQ for staining 10–80 islets in preliminary experiments was 60 μmol/L (data not shown).
- Measurement of zinc/TSQ fluorescence of rat islets with added exocrine tissue. For this experiment, the steps in the fifth experiment were repeated, but known amounts of exocrine tissue were added to the islet samples, and the fluorescence was measured.
- Measurement of zinc/TSQ fluorescence of human islets. Samples of 10–80 handpicked islets of uniform 250 μm diameter were sonicated, and the fluorescence was measured by excitation at 365 nm and emission at 480 nm. The background fluorescence caused by 60 μL TSQ alone in zinc-free distilled water then was subtracted.

RESULTS

The results of the seven experiments are as follows:

- Measurement of rat islet auto-fluorescence. We found a linear correlation between the islet number and fluorescence at excitation 280 nm and emission at 345 nm wavelengths, $r = 0.965$, $P < 0.001$, $n = 15$.
- Measurement of zinc content (fluorescence) of DMSO. Fluorescence of added DMSO was negligible in the range 10–150 μL.

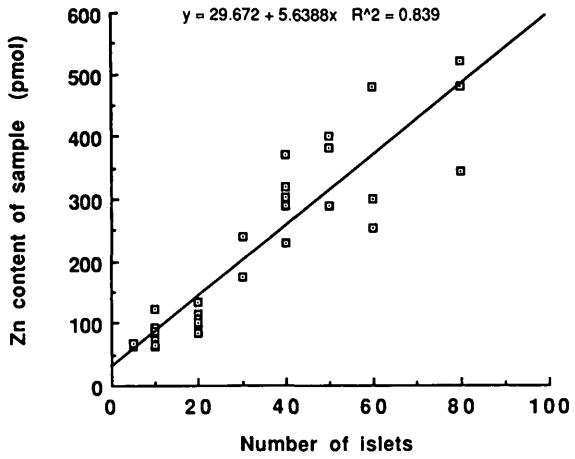

FIG. 1. Correlation between rat islet zinc content measured by EAAS and number of islets (of uniform diameter 250 μm), $n = 31$, $r = 0.917$.

- Measurement of zinc, protein, and insulin content of rat islets. Rat islet zinc was measured by EAAS. Rat islet zinc could be measured in samples containing 10–80 islets, and showed a direct correlation with islet number, $r = 0.917$, $P < 0.001$, $n = 31$ (Fig. 1). Islet protein could be measured in samples containing 10–80 rat islets, and showed a direct correlation with islet number, $r = 0.829$, $P < 0.001$, $n = 20$ (Fig. 2). Fair correlation between the islet number and insulin was detected (Fig. 3), $n = 23$, $r = 0.726$, $P < 0.001$. Fair correlation between insulin and zinc content also was detected in the samples, $n = 23$, $r = 0.716$, $P < 0.001$ (Fig. 4).
- Measurement of the zinc and protein content of rat exocrine tissue. The zinc content relative to protein content was significantly higher in islet than in exocrine tissue, 17.5 ± 7.19, SD; $n = 37$ vs. 3.62 ± 3.06, SD; $n = 42$; $P < 0.001$ (Fig. 5).
- Measurement of zinc/TSQ fluorescence of rat islets. The zinc/TSQ fluorescence showed a linear correlation between islet number and fluorescence, $r = 0.984$, $n = 25$, $P < 0.001$ (Fig. 6).
- Measurement of zinc/TSQ fluorescence of rat islets with added exocrine tissue. Figure 7 shows the effect of adding 10 and 50% exocrine tissue to rat islets. Fluorescence continued to show an excellent correlation with islet number. A small but significant additive fluorescence attributable to the exocrine tissue increased as exocrine tissue increased but was constant at a given concentration.
- Measurement of zinc/TSQ fluorescence of human islets. The zinc/TSQ fluorescence showed a linear correlation between islet number and fluorescence, $r = 0.97$, $n = 10$, $P < 0.001$ (Fig. 8).

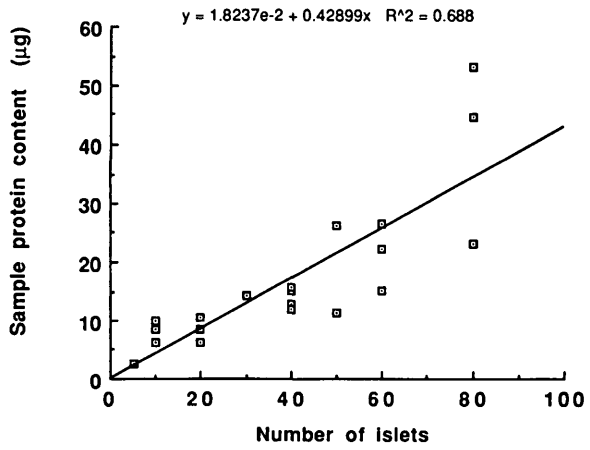

FIG. 2. Correlation between rat islet protein content and number of islets (of uniform diameter 250 μm), $n = 20$, $r = 0.829$.

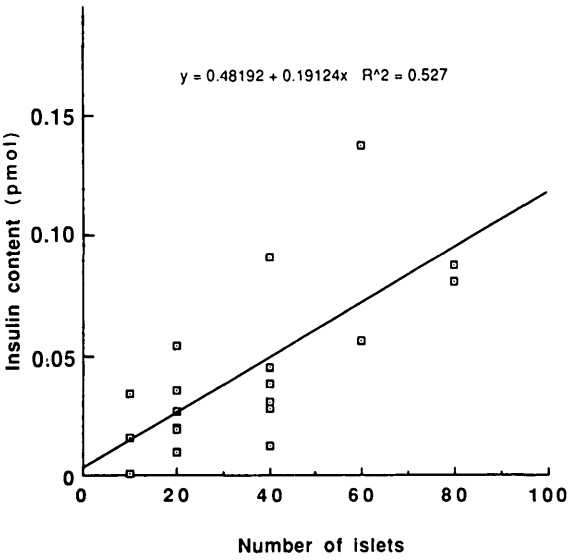

FIG. 3. Correlation between rat islet insulin content and islet number (of uniform diameter 250 μm), $n = 23$, $r = 0.726$, $P < 0.001$.

DISCUSSION

To develop the techniques of islet transplantation to the point of clinical efficiency, it is essential to have strict quality control in each step, including organ procurement and transport, cold storage, islet isolation, purification,

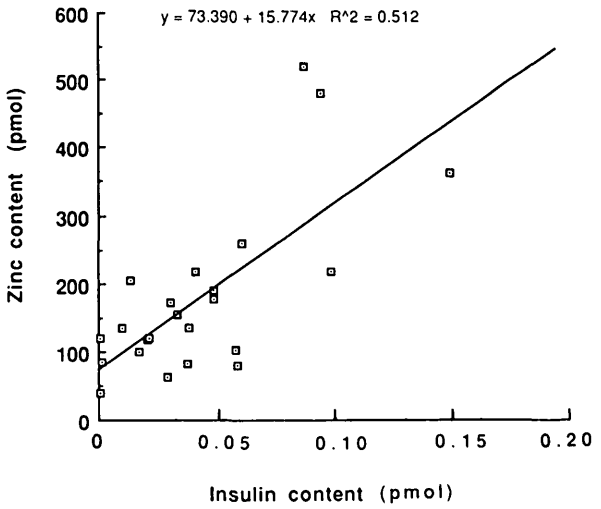

FIG. 4. Correlation between rat islet zinc content and insulin content in samples of 10–80 islets (of uniform diameter 250 μm), $n = 23$, $r = 0.716$, $P < 0.001$.

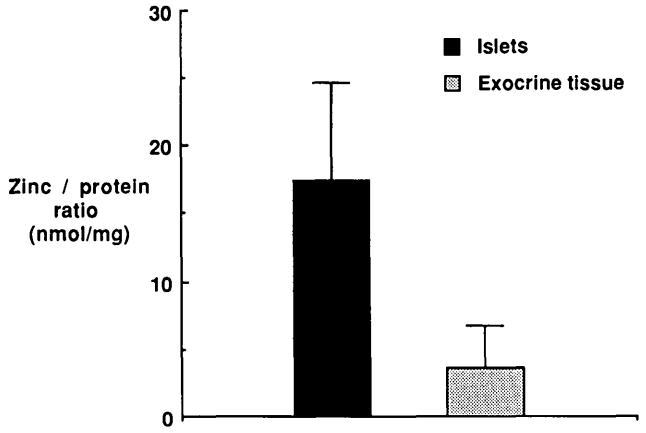

FIG. 5. Comparison of the zinc/protein ratio of rat islets (n = 37) and exocrine tissue (n = 42).

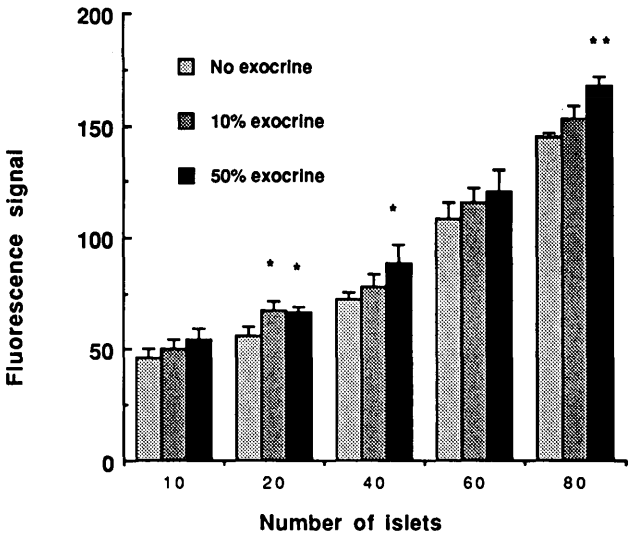

FIG. 7. Effect of adding exocrine tissue on the TSQ fluorescence of rat islets (n = 3 for each number of islets, significance tests vs. no exocrine tissue: *P < 0.05, **P < 0.002).

sterility and documentation of insulin levels, C-peptide and glycated hemoglobin levels, before and after transplantation (30). Over the years, a number of techniques for islet isolation and purification have been developed, including density gradients (8), cell sorter (31), culture (32), and magnetic microspheres coated with antiacinar antibodies (33) to obtain the maximum yield of pure intact islets. Of these procedures, density gradients (Ficoll, BSA, dextran) are used by most researchers (1). The yield obtained from a single pancreas is still not sufficient to reverse insulin dependence in the clinical situation. As the status of islet transplantation becomes firmly established, and more centers become involved in research and clinical trials, a method of comparing islet yields is necessary to developing better protocols for islet isolation.

Hesse et al. (34) proposed using the insulin/amylase ratio to express the islet yield and purity of islet preparations in which the islet mass of the tissue was estimated by measuring insulin content of an aliquot. The degree of exocrine contamination was estimated similarly by assaying the amylase content in the sample. This proposal was ingenious; however, some flaws exist. For example, it has not been shown that the measured insulin represents the islet-insulin alone because a significant amount of insulin probably is released during the process of digestion, and some insulin also is attached to the exocrine tissue during the isolation procedure. In addition, measuring insulin by RIA takes time, and the results would not be readily available before transplantation. Insulin also may be digested variably by enzymes during the incubation necessary for islet isolation, and the activity of amylase also may be affected by interaction with other enzymes during incubation. Most groups have not adopted this method for estimating islet yield.

Other methods of estimating islet yield used a simple count of unstained islets (12). However, this method led to an over-estimate of yields, as clumps of exocrine tissue and lymph nodes could be counted also (14). The introduction of dithizone (35,27), which is zinc specific and stains islets red, allowed positive identification of

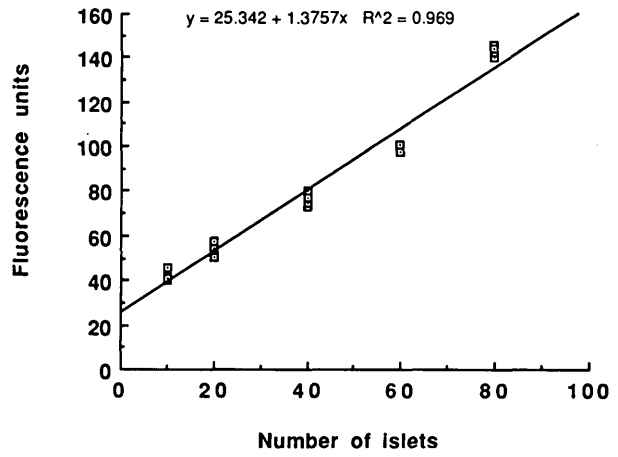

FIG. 6. Correlation between rat islet TSQ fluorescence and islet number (of uniform diameter 250 μm), n = 25, r = 0.984.

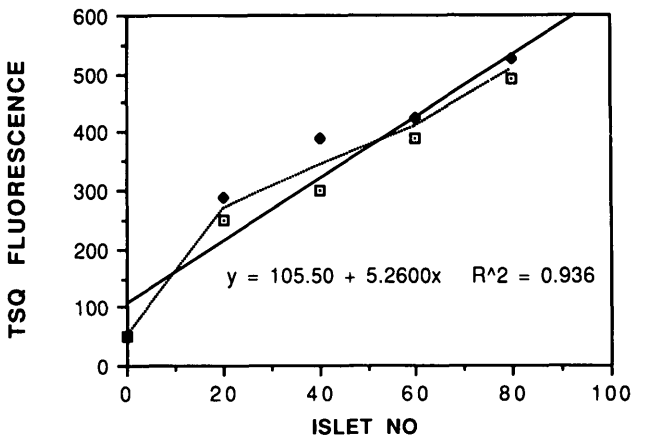

FIG. 8. Correlation between human islet TSQ fluorescence and islet number (of uniform diameter 250 μm). Results using islets from two donors (◆ and ⊡) are shown, with the dotted line corresponding to mean value, and the solid line being the best-fit correlation, n = 10, r = 0.936, P = <0.001.

islets viewed under the microscope. The total count method was efficient in the rodent, however, in humans—with more than a million islets (36)—a sampling technique was necessary. Some researchers have used a technique in which the pancreatic digest was suspended in a 10 ml syringe and continuously agitated; 0.1 mL × 5 samples were taken, and an estimate of the final yield was made (12,14). However, any sampling method introduced an error inversely proportional to the square root of the numbers counted.

The realization that islets are spheres and that a simple count of islets ranging in size from fragments <50 μm to >500 μm in diameter was not accurate led to the method currently favored by most researchers, which is for two independent observers to size each islet sphere in the sample using a graticule eyepiece. The final count then is estimated from the sample mean, and a correction factor of 0.125 expresses the fraction of the islet volume relative to a 150 μm diameter spherical islet (37,14). Thus, the final islet yield would be expressed as 150 μm islet equivalents. Although this method is certainly an improvement, it is not optimal because potential subjective error in accurately sizing each islet sphere still remains, and the assumption that all islets are spherical is certainly incorrect. The adoption of computerized digital analysis to count and size the islets may be the ultimate test for sampling and quantification (Rajotte et al., unpublished observations), but the instrumentation and software is not widely available.

Numerous researchers have shown that zinc content correlates with insulin in the islet. Maske (38) reported a correlation between zinc and insulin secretion in the islet by demonstrating that administration of either glucose or ephinephrine to rabbits decreased the histological staining for zinc in the β-cells of the rat. Bander and Schesmer (39) showed that sulphonylurea compounds administered to rats in vivo caused a loss of histochemically detectable zinc that was parallel to β-degranulation. Toroptsev et al. (40) investigated the zinc content of the A- and B-cells of the islets in different functional states in the rabbit, hamster, mouse, dog, and rat. They determined zinc content by a histochemical method and demonstrated that zinc was concentrated in the B-cells. However, they also found some zinc in the A-cells but in much smaller quantities, and noted that the zinc content of the islets in diabetic rabbits was reduced greatly; whereas starvation caused an accumulation of zinc, and the administration of glucose resulted in a sharp decline of zinc from the B-cells.

The data presented here (Fig. 3) support the validity of the correlation between insulin and islet number, however, this correlation is not as strong as that between zinc and islet number.

This study shows that the zinc content of islets can be measured accurately by EAAS and is barely affected by exocrine contamination in samples of 10–80 islets. The zinc content in islet samples correlated well with the islet yield and islet protein.

However, EAAS is not a practical technique in the transplantation setting. To simplify the zinc measurement, we developed a method using TSQ, a zinc binding fluorescent dye. TSQ has been used previously to stain cells with a high zinc content in tissues such as the pancreas, small intestine, and prostate (41). Frederickson et al. (42) also localized zinc in cells within the brain using TSQ. This group also showed that TSQ can be used for quantitative estimates of CNS zinc pools and qualitative studies of localization, and that TSQ is especially suitable for staining axon button zinc in the brain. We now have shown that TSQ dissolves completely in DMSO at room temperature, and that the peak excitation/emission wavelengths for TSQ are 365/480 nm when dissolved in DMSO. Currently, we also are exploring the use of TSQ for sorting pancreatic islets for islet isolation and purification. (R.M.J., unpublished observations).

These results show that TSQ/zinc fluorescence can be used for zinc estimation and quantifying islets. The additional fluorescence signal from exocrine tissue, although significant, was small. With 50% exocrine tissue, the additional fluorescence was only 16%, and, in practice, the contribution from exocrine tissue would be much lower. If necessary, it would be possible to produce an algorithm to allow for known exocrine contamination. The results need to be extended for islets of other species, but the preliminary results with human islets from two donors are encouraging. The fluorescence intensity obtained from human islets (Fig. 8) was higher than that obtained from rat islets of the same diameter for all numbers of islets measured (Fig. 6). This suggests that the human islets contained more zinc than rat islets, a finding substantiated by measurement using EAAS (data not shown).

The measurement of the small quantities of zinc within islets by TSQ fluorescence is complicated by the need to ensure purity of the solutions used, not only to exclude zinc contamination but any other metals that may induce fluorescence. In addition, we have realized recently that pH may affect the fluorescence intensity (data not shown); indeed, pH difference may have accounted for some, but not all, of the increased fluorescence intensity seen with human islets (Fig. 8). We intend to introduce a buffer in future experiments to stabilize the pH. It also has become apparent that to ensure comparability between measurements in different laboratories, routine calibration of the system using a zinc chloride constant (500 pmol is suggested) would be desirable, and this also will be included in future experiments.

Islet zinc measurement by TSQ fluorescence is likely to be used principally in comparison of human islet yields between laboratories. Obviously, for this purpose it is vital to show that the zinc content of human islets is similar among different pancreases. Our findings in two human pancreases examined are encouraging (Fig. 8), but require confirmation in larger numbers, and such experiments are planned.

TSQ/zinc fluorescence is a sensitive, rapid, cheap, and reproducible method for islet quantification. Its only major drawback is the need for zinc-free reagents and glassware. However, when the appropriate procedures for achieving these conditions are followed, the reproducibility of fluorescence measurements is good, owing to the simplicity of the method.

ACKNOWLEDGMENTS

This work was funded by the British Diabetic Association, the Medical Research Council UK, and the Juvenile Diabetes Foundation (International).

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