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Comparison of insulin responses in experiments using pooled mice islets versus islets from individual animals in the study of diabetes

Stig Eric Dyrskog[a,*], Mogens Erlandsen[b], Jianguo Chen[a], Jing Hong[a], Reziwanggu Abudula[a], Kjeld Hermansen[a]

[a]*Department of Endocrinology and Metabolism C, Aarhus Sygehus THG, Aarhus University Hospital, DK-8000 Aarhus C, Denmark*
[b]*Department of Biostatistics, Aarhus University, DK-8000 Aarhus C, Denmark*

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Abstract

Isolated islets of Langerhans have been widely used to clarify the beta-cell function and insulin secretion in normal and diabetic states. Most of these studies have applied a pooled-islet design using isolated islets from a number of animals. However, to our knowledge, no previous studies have explored the consequences of using the pooled-islet design. From a statistical point, it may be preferable to use a design with islets from only one animal at a time under different experimental conditions and subsequently to repeat the experiments with islets from other animals. The present study compares these 2 designs in terms of their insulin response to glucose and arginine. We found higher insulin responses to glucose and arginine in pooled islets than in islets from individual animals, but differences between different stimuli were comparable between the 2 designs. However, the SEs on these differences are much smaller in the design with pooled islets than in the separate-islet design, and, consequently, corresponding *P* values will be much smaller. The statistical analysis of the design with islet separated per animal can explain this discrepancy because it identifies a non-ignorable random variation between animals compared with the variation within animals. In statistical terms, this implies a positive correlation between islets from the same animal. Ignoring this fact in the design with pooled islets overestimates the degrees of freedom and hence underestimates the SEs on the mean responses. This will in turn produce too small *P* values when using statistical tests to compare mean responses to different stimuli.

1. Introduction

The discovery of Lacy and Kostianovsky [1] of a method for isolation of intact islets from rodents sparked a period of several decades where isolated islets of Langerhans were used for clarifying the beta-cell function and insulin secretion in normal and diabetic states. In many of these studies, researchers applied pools of islets collected from different animals, that is, the pooled-islet design [2-5]. This practice enjoys the advantage of providing a sufficient number of islets for a large experiment. However, the pooling of tissue from different, although genetically related, animals may have drawbacks. Firstly, the islets may only represent one or a few animals. Secondly, islets from different animals may show large interindividual differences in biological responses.

Analyzing data from such experiments, many researchers have applied statistical methods assuming that data were independent sets of observations with identical variance [2-5]. However, data obtained in experiments with the pooled-islet design harbor an unknown mixture of variation between and within animals because the design can only handle one single (common) source of variation, that is, random variation among islets in the pool.

If the interindividual variation is nonnegligible, analysis of such pooled data with standard statistical methods will violate one of the fundamental assumptions of statistically independent observations.

Unfortunately, there is no simple way to separate and quantify the 2 sources of variation with the pooled-islet design. Advantages may be gained from applying a separated islet design, that is, using islets from a single animal at a time under different experimental conditions and subsequently repeating the experiments with islets from other animals. The proper way of analyzing such a design is

* Corresponding author.
E-mail address: stig.dyrskog@ki.au.dk (S.E. Dyrskog).

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by analysis of variance with variation between animals as a random effect (variance component) and different experimental stimuli as fixed effects.

The present study aims to evaluate the possible differences between results obtained with the 2 designs. To our knowledge, this is the first study seeking to clarify this fundamental and important question associated with studies of isolated islets of Langerhans.

2. Materials and methods

2.1. Animals and islet isolation

Eight adult female NMRI mice (Bomholtgaard, Ry, Denmark) weighing 20 to 25 g were used. The animals received standard pellet diet and tap water ad libitum before the experiments. Islets were isolated by the modified collagenase digestion technique [1,5] and kept separately for each of the animals they derived from in the first part of the experiment. In brief, the animals were anesthetized with pentobarbital (50 mg/kg IP), and a midline laparotomy was performed. We filled the pancreata retrogradely with 3 mL ice-cold Hanks balanced salt solution (Sigma Chemical, St Louis, MO) supplemented with 0.3 mg/mL collagenase P (Boehringer Mannheim, Mannheim, Germany) through the pancreatic duct. Subsequently, we removed the pancreata and incubated them separately for 19 minutes at 37°C. After rinsing in Hanks balanced salt solution (3 times), all detectable islets were handpicked under a stereomicroscope and incubated overnight at 37°C and 5% CO_2/95% normal atmosphere in 10 mL RPMI 1640. The latter contained 11.1 mmol/L glucose supplemented with 10% fetal calf serum, 2.06 mmol/L L-glutamate, 100 IU/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (all GIBCO BRL, Paisley, UK). After overnight culture, we rinsed the islets twice in a modified Krebs-Ringer buffer supplemented with 3.3 mmol/L glucose and 0.1% bovine serum albumin (Sigma). All animal experiments were performed in accordance with the Danish Council on Animal Care.

2.2. Experimental setup

The experiment was designed to compare the usual pooled design to the expected preferable individual animal design. As described above, all detectable islets were collected and kept as a group from each animal. After overnight incubation, we divided the group of islets from each animal into 2 groups (groups A and B) by repeatedly picking pairs of equally sized islets, one for each group. We kept the first group of islets (group A) separate for each individual animal (separate-islet design), whereas islets from group B of all of the animals were pooled (pooled-islet design) for 1 hour. There was a surplus of islets in both designs. Subsequently, the secretion studies were carried out. We applied the design with pooled islet to simulate experiments where pancreata from individual animals are pooled early in the isolation process.

After 1 hour of preincubation at 3.3 mmol/L glucose, islets were transferred into 8-well racks and incubated for 1 hour with experimental reagents containing 3.3 mmol/L glucose, 16.7 mmol/L glucose, or 16.7 mmol/L glucose + 10 mmol/L L-arginine.

We added 1 islet and 100 μL experimental reagent to each well in 8-well racks. We carried out the experiments in parallel, using islets from individual animals (separate-islets design) and from the group of pooled islets (pooled-islets design) for each condition. As described, we collected equally sized islets under a stereomicroscope in varying order for the different experimental conditions. There was an excess of islets in both designs. After 1-hour incubation, we collected 50 μL of the incubation medium and froze it for subsequent insulin analysis.

2.3. Assays

Samples were analyzed for insulin using radioimmunoassay technique with a guinea pig anti-porcine insulin antibody (PNILGP4, Novo Nordisk, Bagsvaerd, Denmark), and mono-^{125} I-(Tyr A14)–labeled human insulin (Novo Nordisk) as tracer and rat insulin (Novo Nordisk) as

Table 1
Insulin concentrations from experiments with pooled-islet design and experiments performed with islets from separate animals (variance component model)

	Stimuli	n	Pooled design			n	Separated design					*P*
			Mean	SEM	SD (s)		Mean	SEM	SDs			
									Between (s_B)	Within (s_W)	Total (s_T)	
Insulin	3.3 mmol/L glucose	63	0.799	0.064	0.50	64	0.487	0.046	0.12	0.16	0.20	.0004
	16.7 mmol/L glucose	64	34.730	2.624	21.0	64	17.470	3.454	9.18	9.47	13.2	.002
	16.7 mmol/L glucose + 10 mmol/L L-arginine	64	62.529	2.490	19.9	60	46.206	4.867	13.1	11.7	17.5	.017
Log(ins)	3.3 mmol/L glucose	63	−0.331	0.053	0.42	64	−0.791	0.092	0.24	0.27	0.37	.002
	16.7 mmol/L glucose	64	3.351	0.084	0.67	64	2.523	0.223	0.58	0.70	0.91	.010
	16.7 mmol/L glucose + 10 mmol/L L-arginine	64	4.091	0.037	0.30	60	3.768	0.096	0.25	0.26	0.36	.016

Mean insulin secretions (ng/mL per islet per 60 minutes), SE, and SDs for both designs are shown. In the separate-islets design, SDs are shown for both between and within animals. Total SD ($s_T = \sqrt{s_B^2 + s_W^2}$) can be compared with the SD in the pooled design (s). The P value corresponds to an unpaired t test on the 2 mean responses from the different setups. n indicates the total number of single-islet experiments in both designs for each condition; Log(ins), logarithmic transformation of data to approximate to the normal distribution.

Table 2
Bartlett test applied for equal variance in data from experiments using islets from separate animals

Stimuli	Bartlett test, *P*	
	Insulin	Log (insulin)
3.3 mmol/L glucose	<.0001	.0064
16.7 mmol/L glucose	.0208	.0342
16.7 mmol/L glucose + 10 mmol/L L-arginine	.0805	.0628

P values are shown for absolute insulin data and after logarithmic transformation to approximate to the normal distribution.

standard. We separated free and bound radioactivity using ethanol [6]. Inter- and intra-assay variations were less than 10%.

2.4. Statistical analysis

Data from the 2 designs were analyzed differently based on different statistical assumptions in the 2 experimental setups. In the separate-islets design, analysis of variance was used with variation between animals as a random factor (variance component model). We compared the mean insulin responses from the 2 different designs, at each test situation using an unpaired *t* test with unequal variances.

In the pooled-islets design, we calculated SEs for the effects of each experimental condition as an ordinary SEM. In addition, we used the magnitude of the SEMs to evaluate the 2 designs.

Comparisons of different stimulations within each design were done using Satterthwaite approximation to the degrees of freedom because the variances at different stimulations were unequal. In the separate-islets design, we further compared at each stimuli the intraindividual variation between animals using Bartlett test [7]. To obtain a better approximation to the normal distribution, we analyzed data on a log scale. For statistical analysis, we applied SAS (v.8.2. proc mixed, SAS Institute, Cary, NC).

3. Results

Table 1 shows the mean insulin concentrations and corresponding SEMs for the 2 designs and the SDs on the variance components. As expected, insulin release rose in response to a rise in glucose concentration from 3.3 to 16.7 mmol/L. It further rose upon addition of 10 mmol/L arginine in the presence of 16.7 mmol/L glucose in both experimental settings. Interestingly, we observed higher insulin responses in absolute values with pooled islets than with islets from individual animals both at high glucose and after arginine supplementation.

In the design with separate islets the variation within an animal can be compared with the variation within the other animals receiving identical stimuli. Apparently, there is some heterogeneity among the animals, especially at the low glucose (Table 2). The distribution of insulin responses from each individual animal was skewed to the right (data not shown), but logarithmic transformation failed to eliminate the problem with variance heterogeneity. Table 3 shows mean differences in insulin responses, between different stimuli with glucose 16.7 mmol/L as reference in both designs. As can bee seen, the relative differences (back-transformed differences on the logarithmic scale) (Table 3) in insulin response is less different compared with the absolute levels (Table 1) between the 2 designs. The pooled model assumes a higher number of degrees of freedom, reflected in the lower SE on the differences, and the more narrow confidence intervals.

4. Discussion

This study addresses a principal problem in experiments using isolated islets of Langerhans. Many researchers have used islets pooled from a number of animals to gather sufficient material to conduct large and complex series of experiments. This approach rests on the assumption that secretory responses from islets deriving from different animals are comparable because the animals are from the same strain. In the present study, we compared insulin responses obtained using both the pooled and the individual islet design.

As expected, the increment in insulin secretion in response to high glucose was further augmented upon addition of arginine in both experimental setups. We divided the islets from each animal into 2 groups by repeatedly

Table 3
Standard errors and confidence intervals on estimated differences between different stimuli with 16.7 mmol/L glucose as reference, analyzed on ln-transformed data

Design	Stimuli	ln-transformed					Back-transformed (exp)		
		Diff	SE (Diff)	DF*	95% CI		Diff	95% CI	
					Lower	Upper		Lower	Upper
Separated	3.3 mmol/L glucose	−3.314	0.258	9.33	−3.895	−2.733	0.036	0.020	0.065
	16.7 mmol/L glucose	Reference							
	16.7 mmol/L glucose +10 mmol/L L-arginine	1.244	0.260	9.52	0.662	1.827	3.47	1.94	6.22
Pooled	3.3 mmol/L glucose	−3.682	0.099	105	−3.880	−3.485	0.025	0.021	0.031
	16.7 mmol/L glucose	Reference							
	16.7 mmol/L glucose +10 mmol/L L-arginine	0.740	0.092	86.5	0.557	0.923	2.10	1.75	2.52

Diff indicates difference between reference (16.7 mmol/L glucose) response and responses to other stimulations, respectively; Diff (back-transformed), the ratio between the median for the stimuli and the reference; SE (Diff), SE on differences; CI, confidence interval on differences; DF*, Satterthwaite approximation to degrees of freedom (DF) and this was used because variances are unequal across the different stimulations.

selecting pairs of equally sized islets, one for each design. All animals thereby contributed to the pool of islets with the same number and size of islets as in the group of islets used for the separate-islet design. A rather puzzling phenomenon was that the glucose-induced insulin release was clearly higher in the pooled rather than in the separate-islet design group. This may be due to a selection bias, that is, the islets picked for secretion studies in the pooled group tended to be larger than in the separate-islet design group. When seeking equal-sized islets for the experiment, the number of available larger islets may be small when sampling from individual animals, whereas the pooling procedure will increase the number of available large islets and thereby the probability of sampling larger islets. Measurements of total islet insulin content and/or total islet DNA content could have provided important further information on this issue. Unfortunately, these measurements were not carried out because the finding was unexpected. Previously, it has been demonstrated that the insulin secretion rates are positively correlated to increasing size of islets [8]. Thus, the differences in insulin levels between the 2 designs could reflect a bias in the sampling procedure.

It is evident from Table 1 that there is much variation in insulin response in experiments using the separate-islet design. However, the separated-islets design includes a pool per animal, and this practice may have introduced a subsequent "pool-to-pool" variation. Our design was unable to distinguish between the 2 sources of variation: "pool to pool" and "animal to animal." In the following we refer to these sources of variation as "variation between animals," as we presume that the latter to be the dominant.

Variation between animals was large, and in the presence of both high glucose and arginine, we even detected a higher between animal than within-animal variation. This may indicate that the animals differ more than previously presumed. Bartlett test for equality of variances further showed that variations in data originating from different animals were not identical, and logarithmic transformation failed to solve this (Table 2).

As seen from Table 1, the separate-islet design displayed a smaller within-animal variation than residual variation from the pooled-islet design. From a statistical point of view, this was to be expected because the latter assumes that the between-animal variation is negligible compared with the within-animal variation. In contrast, in the separate-islet design, these 2 sources of variation were of equal magnitude. The presence of a non-ignorable variation between animals implies a positive correlation between islets from the same animal. Thus, our data, with design-related limitations, raise questions regarding the practice of analyzing data from pooled-islet designs as statistically independent observations. Ignoring this fact in the design with pooled islets will overestimate the degrees of freedom and hence underestimate the SEs on the mean responses, which in turn will produce too small P values when using statistical tests to compare responses to different stimuli.

It is generally taken for granted that islets from different animals can be pooled and that the results are not systematically influenced by this procedure. Our findings call this assumption in question. A possible pool-to-pool variation from the different animals may overestimate the impact of the between-animal variation in our data and this calls for attention in future studies.

Further studies are needed with other stimuli, for example, second messengers such as Ca^{2+} and cyclic adenosine monophosphate as well as measurements of total islet insulin content and/or total islet DNA content, to get a more accurate explanation for the differences between pooled and individual setups.

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