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Pancreatic Islet Stereology: Estimation of Beta Cells Mass

Estereología de los Islotes Pancreáticos: Estimación de la Masa de Células Beta

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SUMMARY: Obesity and its comorbidities are becoming epidemic in the Western world. Beta cell mass estimation is an important indicator to track the progression of insulin resistance/type 2 diabetes, particularly in experimental studies, where it can be performed with stereological tools in an unbiased way. In this work, we present a simple protocol that can contribute to doing the practice of estimating the mass of beta cells more frequent and reproducible. As with any quantitative study, the necessary precautions regarding sampling and randomness must be respected.

KEY WORDS: Pancreatic islet; Beta cell; Stereology; Obesity; Cell biology.

INTRODUCTION

The obesity epidemic in the world is associated with diabetes mellitus, coronary heart disease, certain forms of cancer, and sleep-breathing disorders, in which insulin resistance/type 2 diabetes (T2D) is relevant (Kopelman, 2000), as demonstrated in the Hispanic population in the United States (Aguayo-Mazzucato *et al*., 2019). Also, the exposure of mothers' diabetic pregnancies or gestational impaired glucose tolerance leads to inter-generational amplification of T2D risk mediated through prenatal exposures (Dyck *et al*., 2019). The maternal vitamin D-restricted diet modifies the development of the pancreas of the offspring, leading to islet remodeling and altered insulin-signaling pathway. The decrease of pancreatic and duodenal homeobox 1 (PDX-1) is probably significant to the changes in the beta-cell mass and insulin secretion in adulthood (Maia-Ceciliano *et al*., 2016). Empowering community diabetes mellitus screening programs targeting the environment, social gradients, and cultural norms while engaging in preventive interventions are recommended (Cuschieri & Grech, 2019).

Pancreatic beta cells are responsible for maintaining the body's glucose levels within a very narrow range; their population is dynamic, with compensatory changes to maintain euglycemia. Throughout the lifetime of a mammal, low levels of beta-cell replication and apoptosis are balanced and result in a slowly increasing mass of beta cells (Bonner-Weir, 2000). One way to evaluate the evolution of insulin resistance / T2D, as well as the efficiency of therapeutic procedures, is by estimating the beta cell mass in the pancreatic islet (Frantz *et al*., 2011; Mandarim-de-Lacerda, 2019). The progressive loss of pancreatic beta-cell mass that occurs in T2D is a primary factor driving efforts to identify strategies for effectively increasing, enhancing or restoring beta-cell mass as constant stimulation of beta-cell proliferation has remained a challenge (Aamodt & Powers, 2017). Islet neuropeptide Y receptors are promising targets for the preservation of beta-cell mass and targeting these receptors could help to maintain beta-cell mass in both type 1 and type 2 diabetes, and may also be useful for improving islet transplantation outcomes (Franklin *et al*., 2018).

A relevant tool in experimental studies is the use of quantitative methods in morphology (morphometry and stereology) (Mandarim-de-Lacerda & Del-Sol, 2017), and islet and beta cell mass have been stereologically studied (Bock *et al*., 2003a,b).

However, for this method to be more acceptable to researchers of metabolic disorders of glucose homeostasis and pancreatic islet, the present manuscript attempts to standardize a quick and easy method of execution of estimating the mass of beta cells using design-based stereology.

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Example with the mouse pancreas. Whenever we do a quantitative investigation, we must keep in mind the statistical requirements. Is the sample representative of the population? If a second sample from the same population is taken, will the results be consistent with the first sample? It is easy to understand that we should always do a 'pilot' study to define the best sample to be examined, the one that best represents the population. Also, increasing the number of individuals in the sample is a general recommendation that improves statistical analysis (the improvement of the sample size usually is related to a better chance of being closest to the population) (Mandarim-de-Lacerda & del-Sol).

Pancreas: volume and mass estimation: At the sacrifice of the animal, the pancreas should be carefully removed, with minimal fat adjoining. The volume of the pancreas should be obtained with the organ immersed in physiological solution (Scherle's method) in a container placed on the scale. Thus, both the volume and mass of the pancreas are obtained at the same time (Scherle, 1970). Alternatively, the volume of the pancreas can be estimated by the Cavalieri principle in serial sections of the pancreas (Mandarim-de-Lacerda, 2003).

Preparation for light microscopy: The pancreas of the mouse should be fixed in a fixative solution for 48 h (4 % w / v, 0.1 M formaldehyde, pH 7.2) and then embedded in paraffin or paraplast plus (the mouse pancreas is small and can be included whole, Sigma-Aldrich Co., St. Louis, MO, USA) to light microscopy or confocal laser scanning microscopy.

Sectioning, staining, and sampling: The block containing the pancreas must be thoroughly sectioned (e.g., with 5 mm of thickness). It is essential to keep all sections, even if only a few cuts are necessary for the study.

The size of the islet should be measured in some sections (a pilot study) in order to determine the spacing of the cuts required for the stereological determinations, fundamental because depending on the experimental design, the islets may vary in size requiring adaptations in the protocol of stereology.

Let us assume that the average islet size in our example is 100 mm. In this case, in order not to consider more than once the same islet, a spacing of 20 cuts should be preserved (since 5 mm x 20 = 100 mm).

For each animal in the sample, the initial section starting the counting of the twentieth section shall be random (e.g., decided by lottery), and from that section, each twenty-first section shall be analyzed.

It is proposed to select three sections at each interval:
a) Staining with hematoxylin and eosin,
b) Staining with anti-insulin antibody,
c) Staining with anti-glucagon antibody (just in case of also determining the a cells mass).

Therefore, the study will be optimized and able to estimate the mass of b cells and the mass of a cells.

Figure 1 shows the sampling of sections in the pancreas of the mouse.

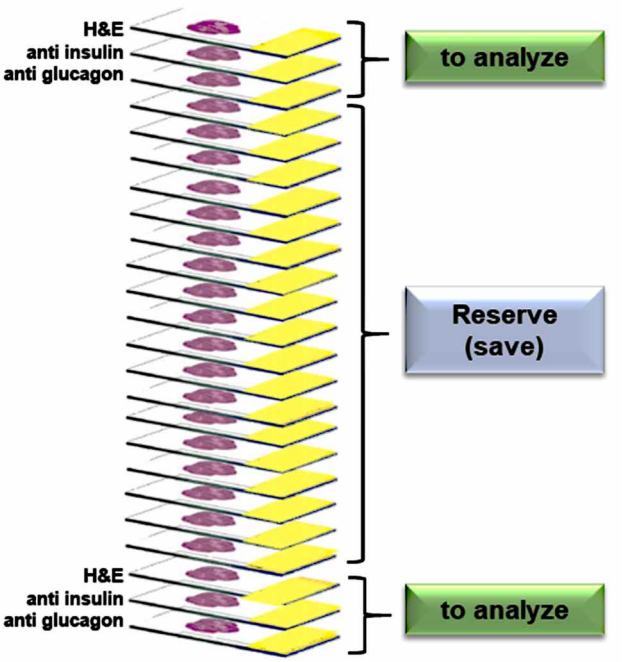

Fig. 1. The scheme illustrates the serial sections of the pancreas of the mouse and the spacing indicated to avoid the analysis of the same islet. To each pancreas (each animal in the group) the beginning of the analysis should start randomly.

Estimating islet β cells mass: The complete sequence of events to estimate the beta cell mass is shown in Figure 2. Steps 1 and 2 were carried out previously.

Islet volume density (Vv [islet, pancreas %): We need to know how much of the pancreas is occupied by the islet, named the islet volume density. There are many ways to determine the islet volume density, but the most practical is by the technique of "point counting" (Mandarim-de-Lacerda & del Sol).

In Figure 3, the islet density in the photomicrograph corresponds to the ratio of the number of points to the islet and the total number of points in the test system (in this

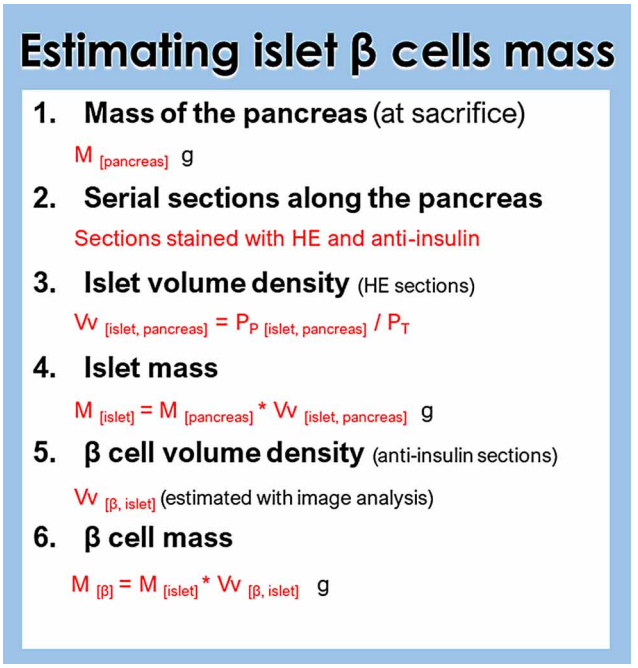

Fig. 2. Panel detailing the steps to be followed for estimating the beta cell mass in the pancreas of the mouse.

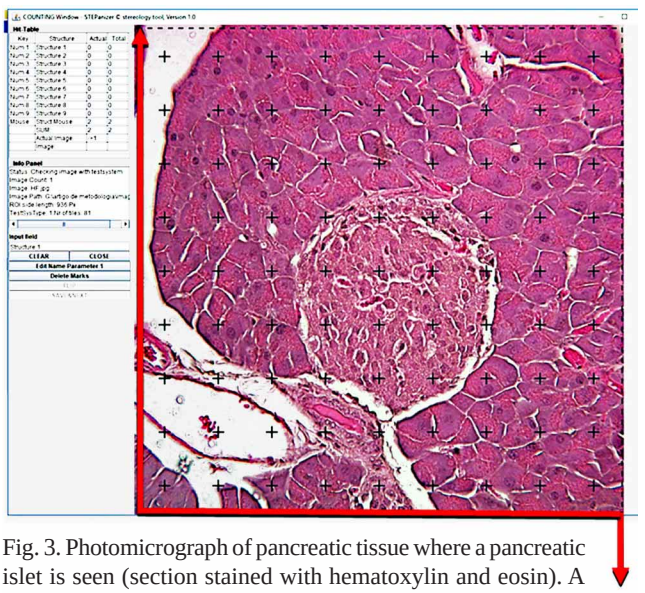

Fig. 3. Photomicrograph of pancreatic tissue where a pancreatic islet is seen (section stained with hematoxylin and eosin). A test point system was superimposed on the photomicrograph to estimate the islet volume density by 'point-counting.'

example we used the Stepanizer web-based application (www.stepanizer.com) to overlap the point system to the pancreas photomicrograph). It should be noted that the photomicrographs of the pancreas must be taken at random, containing islets or not (the islets usually correspond only 1 to 2 % of the pancreas in mammals) (Mandarim-de-Lacerda, 2019).

Islet mass (g): The islet mass is estimated by the product of the islet density in the pancreas and the mass of the pancreas (previously obtained).

β cell volume density (Vv [b, islet] %): Here, we should use sections stained with anti-insulin antibody to ensure that we are quantifying b cells. Thus, image analysis is the practical technique of determining b-cell density in the islet. Figure 4 illustrates this analysis. The islet with immunohistochemistry staining was outlined (the 'are of interest,' AOI tool) and the density threshold selection tool was applied to the insulin-positive area estimating their percentage of the islet (Image pro plus version 7.1 for Windows, Media Cybernetics, Rockville, MD, USA) (Chlipala *et al*., 2019).

β cells mass (g): Finally, the b cell mass can be estimated by the product of the islet mass and the density of b cells.

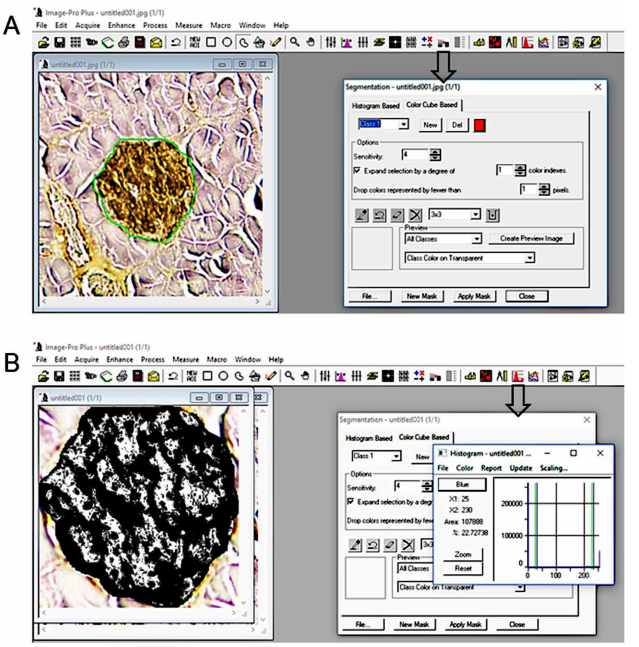

Fig. 4. Print screens of two steps of the estimation of the volume density of b cells by image analysis. A - delimitation of the 'area of interest', B - segmentation of the region with immuno-labeling with anti-insulin antibody and density estimation.

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RESUMEN: La obesidad y sus comorbilidades se están convirtiendo en una epidemia en el mundo occidental. La estimación de la masa de células beta es un indicador importante para rastrear la progresión de la resistencia a la insulina/diabetes tipo 2, particularmente en estudios experimentales, donde se puede realizar con herramientas estereológicas de manera imparcial. En este trabajo presentamos un protocolo simple que puede contribuir a que la práctica de estimar la masa de células beta sea más frecuente y reproducible. Como en cualquier estudio cuantitativo, deben respetarse las precauciones necesarias con respecto al muestreo y la aleatoriedad.

PALABRAS CLAVE: Islote pancreático; Célula beta; Estereologia; Obesidad; Biología celular.

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