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Consequences of a Fat Diet in the Distribution of Minerals within Pancreatic Tissues of Rats and Rabbits

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Abstract: The effects of plasma lipid overload on pancreatic islet function and on mineral imbalance are issues under debate. However, the outcomes may be biased by the different metabolisms of different species. This prospective study evaluated whether a high fat diet intake changed the distribution of physiologically relevant elements within pancreatic endocrine and exocrine tissues of Sprague Dawley rats and New Zealand White rabbits. Nuclear microscopy techniques provided images of the specimen density and structure as well as the elemental distributions and quantification of P, S, Cl, K, Ca, Fe, and Zn using unstained cryosections of pancreas. Our results indicate that pancreatic islets in normal rats and rabbits had lower tissue density and higher Ca, Fe, and Zn content compared to exocrine tissue, and that rabbit islets exhibit the highest Zn content (3,300 μg/g in rabbits versus 510 μg/g in rats). Fat diet intake resulted in large deposits of fat in the pancreas, which modified the density contrast of tissues and also resulted in a twofold decrease of Ca and Zn concentrations in islets of both rats and rabbits. This result indicates that a fat diet leads to a reduction in essential trace element concentrations in pancreas, which in turn may hamper endocrine function.

Key words: zinc, iron, nuclear microscopy, islets of Langerhans, rabbit, rat

Introduction

Regular consumption of an energy enriched diet, including a high content of minerals, fat, cholesterol, and glucose, has been associated with impaired glucose tolerance, hyperinsulinemia, and dyslipidemia. These features may have serious consequences in pancreas, liver, skeletal muscle, and adipose tissue metabolism and are considered risk factors for developing type 2 diabetes mellitus and cardiovascular diseases in humans (Dandona et al., 2005; Pinnick et al., 2008). The continuous excess of glucose and free fatty acids (FFA) in blood induces lipid accumulation in the pancreas, and there is evidence that they play a direct role in pancreas dysfunction (Nolan et al., 2006; Hao et al., 2007).

The pancreas is a complex organ with important endocrine and exocrine functions. The islets of Langerhans account for much of this complexity consisting of endocrine cells with a number of secretory products including glucagon (α-cells) and insulin (β-cells) that regulate metabolism of carbohydrates, lipids, and proteins (Saltiel & Kahn, 2001). Under normal β-cell function, insulin secretion is activated when the glucose levels rise in blood and inhibits glucagon release from α-cells. A fall in blood glucose leads to a pronounced decrease in insulin secretion, activating α-cells to deliver glucagon to the blood. The coordinated secretion of insulin and glucagon regulate carbohydrate, lipid, and protein metabolism (Saltiel & Kahn, 2001). In turn, deprivation of FFA *in vivo* diminishes glucose-stimulated insulin secretion whereas exposure augments it (Stein et al., 1997). Under overload conditions, the intricate interactions between glucose, FFA (Peyot et al., 2009), cholesterol (Hao et al., 2007; Tsuchiya et al., 2010), their uptake, synthesis, and clearance seem to influence β-cell secretory function, compensation for insulin resistance, apoptosis, and ultimately islet dysfunction (Saltiel & Kahn, 2001; El-Assaad et al., 2003; Nolan et al., 2006).

Insulin is stored in granules of β-cells, where it co-crystallizes with Zn. At present it is accepted that insulin release from granule pools involves the dissociation of Zn-insulin dimmers complex (Guyton & Hall, 2000; Eliasson et al., 2008). Evidence is also accumulating on the role of Zn^{2+} ion as a signaling molecule of islet cells. The decrease of Zn^{2+} free ions in the periportal circulation paralleling the fall in insulin seems to provide the signal to α-cell secretion of glucagon (Slucca et al., 2010). However, the effect of β–cell derived Zn^{2+}, exogenous Zn, and the significance of Zn imbalances on islet secretory functions are issues that are far from being clarified (Hao et al., 2007; Zhou et al., 2007; Kawamori et al., 2009).

Although several studies using animal models have contributed to understanding secretion mechanisms, islet access of plasma lipids and minerals such as Zn and the

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effect of these nutrients on pancreatic islet function are issues under debate (Ravier & Rutter, 2005; Zhou et al., 2007; Slucca et al., 2010; Wagner et al., 2010). In addition, nutrient overload and/or deprivation effects may be biased by the species differential metabolism. The recent developments in nuclear microscopy using a focused MeV proton beam, which can provide high-resolution images of tissue morphology and elemental distributions (Ynsa et al., 2009), may significantly contribute to the investigation of mineral contents of tissues. Using proton induced X-ray emission (PIXE) microscopy, the visualization and quantification of the process of mineral transfer through the pancreatic endocrine and exocrine cells under different nutritional conditions can be followed.

In previous studies, carried out under starvation conditions mostly using mice as a model (Juntti-Berggren et al., 1991), the distribution of minerals were investigated. However, the mineral distributions under fat overload were not addressed. The study presented here evaluates the distribution of Zn and other minerals in pancreas Langerhans islets and in surrounding exocrine tissue under excess dietary fat. Two animal models, rabbits (herbivorous) and rats (omnivorous), were used to assess interspecies variability in morphological pancreatic islet features under normal condition and their changes due to a high fat diet.

Material and Methods

Sampling

Two animal models, rabbits and rats, have been chosen for this study. The rabbits were 8-week-old male New Zealand White rabbits with a weight range between 2.2 and 3.2 kg. The rats were 11-week-old male Sprague Dawley rats with a range in weight between 450 and 520 g. A total of 10 rabbits and 10 rats were used in the study distributed into four groups of 5 animals each. They were housed in a temperature (24 + 1°C) and humidity (55 + 3%) controlled room with a 12 h light/dark cycle following the guidelines of the Animal Care and Use Committee of the National University of Singapore and maintained *ad libitum* on laboratory 1 chow (Glen Forrest Stockfeeders, Glen Forrest, WA, Australia; www.specialtyfeeds.com). Animals were fed with a customized high fat diet (rats 4.4 kcal/g; rabbits 3.4 kcal/g) containing 18% fat and 1% cholesterol, which were low in Ca and P to facilitate colon uptake of fat (Bovee-Oudenhoven et al., 1999; Papakonstantinou et al., 2003) during 4 weeks. Control animals were kept under the same environmental conditions and received standard animal diet (rats 3.3 kcal/g; rabbits 2.7 kcal/g) for rat, guinea pig, and rabbits.

Before extraction of the pancreas, all animals were killed by IV injection of sodium pentobarbitone (0.8 mg/kg). Two pancreas portions just below the spleen were removed from each animal. Residual blood of the pancreas was removed from the tissue by flushing with deionized water. The blocks of tissue were then flash frozen in liquid nitrogen and stored at −80°C. Serial sections of 10-μm thicknesses were cut using a Leica CM3050S cryostat set at −17°C with the knife at −22°C. Sections were picked up on gelatin-coated glass sides for hematoxylin and eosin (H&E) staining to identify artifacts, inspect morphology, and define regions of interest. Adjacent unstained sections were picked up on freshly made pioloform film of 0.5 μm mounted on appropriate target holders for nuclear microscopy analysis.

Experimental

The pancreas sections were analyzed by nuclear microprobe to visualize the tissue structure and assess the distribution of elements and their quantification (Breese et al., 1996). The experimental setup of the Centre for Ion Beam Applications (CIBA) of the National University of Singapore (Watt et al., 1994) was used. Several techniques can be operated simultaneously: PIXE for minor and trace element determination, Rutherford backscattering spectrometry (RBS) for incident charge measurement and matrix composition evaluation that are used in elemental concentration calculation, and scanning transmission ion microscopy (STIM) for sample mass density determination. STIM can also provide high-resolution images of tissue structure.

A beam of 2.1 MeV protons focused to a diameter of 1 μm was used. The incident proton beam was at 45° to the sample surface, and the beam current was in the range of 100–300 pA. Transmitted protons, which are collected to form the STIM image, were detected using an ion-implanted silicon charged particle detector placed off beam axis (at 20° to the beam). A lithium-drifted silicon detector at 90° to the beam, with a 300-μm Perpex® filter with 1-mm-diameter central hole to attenuate X-rays from light elements, was used to detect X-rays (PIXE). For RBS analysis, backscattered protons were detected using a silicon particle detector with an active area of 50 mm^2 at a scattering angle of 160° to the beam.

The methodology we employed in this study consisted first of identifying the main structures of the pancreas through density maps (STIM maps), which provide high contrast images of tissue features and, second, selecting regions of interest for detailed imaging, elemental mapping, and quantification. The extraction of spectral data was carried out on selected beam positions and from regions of interest. The analysis and the calculation of quantitative results of X-ray and RBS spectra were done with GUPIX (Maxwell et al., 1989) and SIMNRA (Mayer, 1997) software.

Statistical Analysis

All statistical analyses were performed using the SPSS statistical package (version 18.0, SPSS Inc., Chicago, IL, USA). Means and standard deviations were used as descriptive statistics. Significant changes in elemental concentrations of endocrine and exocrine pancreas and the differences between groups of animals were determined using Mann-Whitney and Kruskal-Wallis nonparametric tests. Differences were considered significant when a confidence interval of 95% was exceeded ($p < 0.05$).

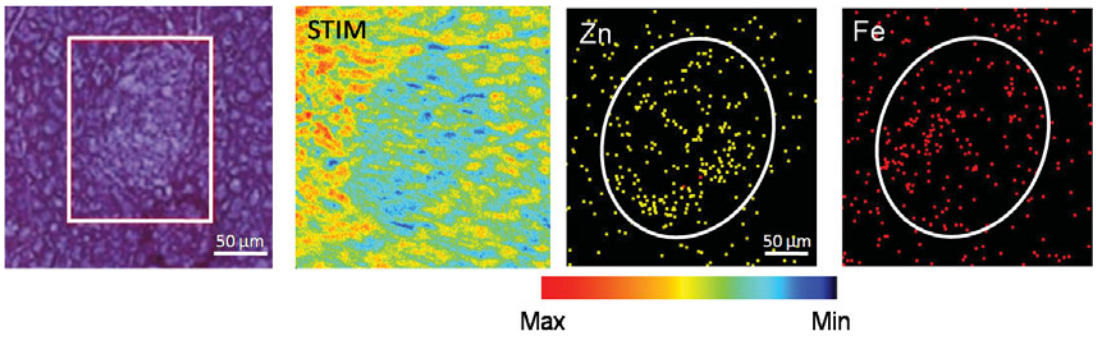

Figure 1. Optical image of an H&E serial stained section of normal rat pancreas, proton STIM map, Zn map, and Fe map. The islet of Langerhans, which can be identified in the optical image (white square) of the stained section, is easily defined in the STIM image. The zinc and Fe distributions are associated with the endocrine pancreas (circle).

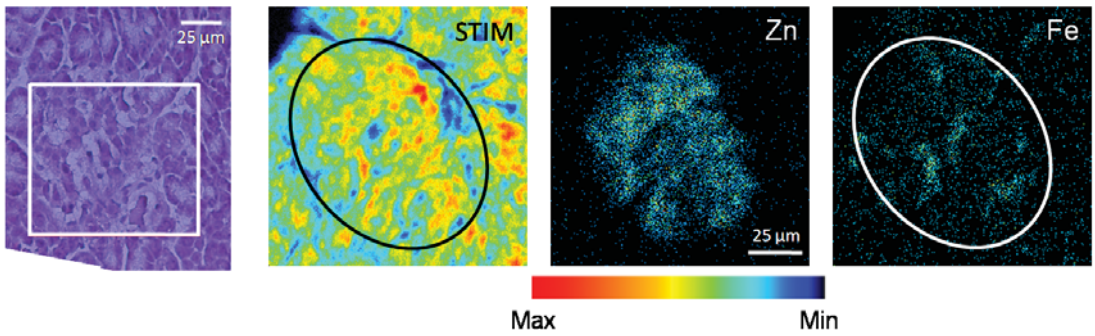

Figure 2. Optical image of an H&E serial stained section of normal rabbit pancreas, proton STIM map, Zn map, and Fe map. The islets of Langerhans, which can be identified in the optical image (white square) of the stained section, is not easily recognized in the STIM image. The zinc and Fe distributions are associated with the endocrine pancreas (circle).

Results

Islet Characterization and Elemental Distribution

The rat islets seem to be larger than rabbit islets, as can be depicted from direct observation of the optical and nuclear microscopy images shown in Figures 1 and 2, although rat pancreas showed fewer islets than rabbit pancreas per unit area. For the five animals analyzed in each group of rats and rabbits fed with normal diet, and for at least two sections from each animal, an area of approximately 5 mm^2 of rat pancreas had an average of two islets, whereas rabbit pancreas had about four islets. While STIM maps indicate the density changes of the unstained sections, PIXE provides the distributions of P, S, Cl, K, Ca, Fe, and Zn. We therefore made use of Fe and Zn imaging to enable us to distinguish endocrine and exocrine regions of pancreas (Figs. 1c, 1d, 2c, 2d). The most significant feature observed in elemental maps is the high Zn concentration associated with the islets of Langerhans. Comparing the corresponding STIM and Zn maps, it is possible to identify the high Zn areas as islets of Langerhans in both rats and rabbits, as depicted in Figures 1c and 2c. The complementary nature of STIM and PIXE therefore proved useful not only in the interpretation of results but also in the location of the regions of interest.

No major differences were found in the distribution of minor elements (i.e., P, S, Cl, and K) between endocrine and exocrine pancreas of control rats and rabbits, under the nonfat diet condition (Fig. 3). Their distribution was uniform, and their concentrations in islets and exocrine pancreas did not differ. On the contrary, the Ca, Fe, and Zn concentrations observed in endocrine and exocrine pancreas were nonuniform. Increased levels of Ca, Fe, and Zn were observed in the pancreatic islets of rabbits and rats (Table 1), and differences compared with the levels of these

Table 1. Ca, Fe, and Zn Mean Concentration and Standard Deviation ($x \pm SD$) of Endocrine and Exocrine Pancreas of Normal (N) and Fat-Diet (FD) Animals.

	Rat		Rabbit	
	N ($x \pm SD$)	FD ($x \pm SD$)	N ($x \pm SD$)	FD ($x \pm SD$)
Ca				
Endocrine	800 ± 140	380 ± 180^a	1100 ± 300	700 ± 300^a
Exocrine	410 ± 50^b	330 ± 110	390 ± 140^b	410 ± 170
Fe				
Endocrine	160 ± 80	91 ± 24	170 ± 90	80 ± 40^a
Exocrine	63 ± 19^b	59 ± 6	86 ± 19^b	70 ± 40
Zn				
Endocrine	510 ± 110^c	190 ± 160^a	$3{,}300 \pm 1{,}200$	$1{,}400 \pm 800^a$
Exocrine	116 ± 13^b	74 ± 13	90 ± 19^b	130 ± 80

[a]Significantly different from controls ($p < 0.05$).
[b]Significantly different from endocrine pancreas within species.
[c]Interspecies difference ($p < 0.05$).

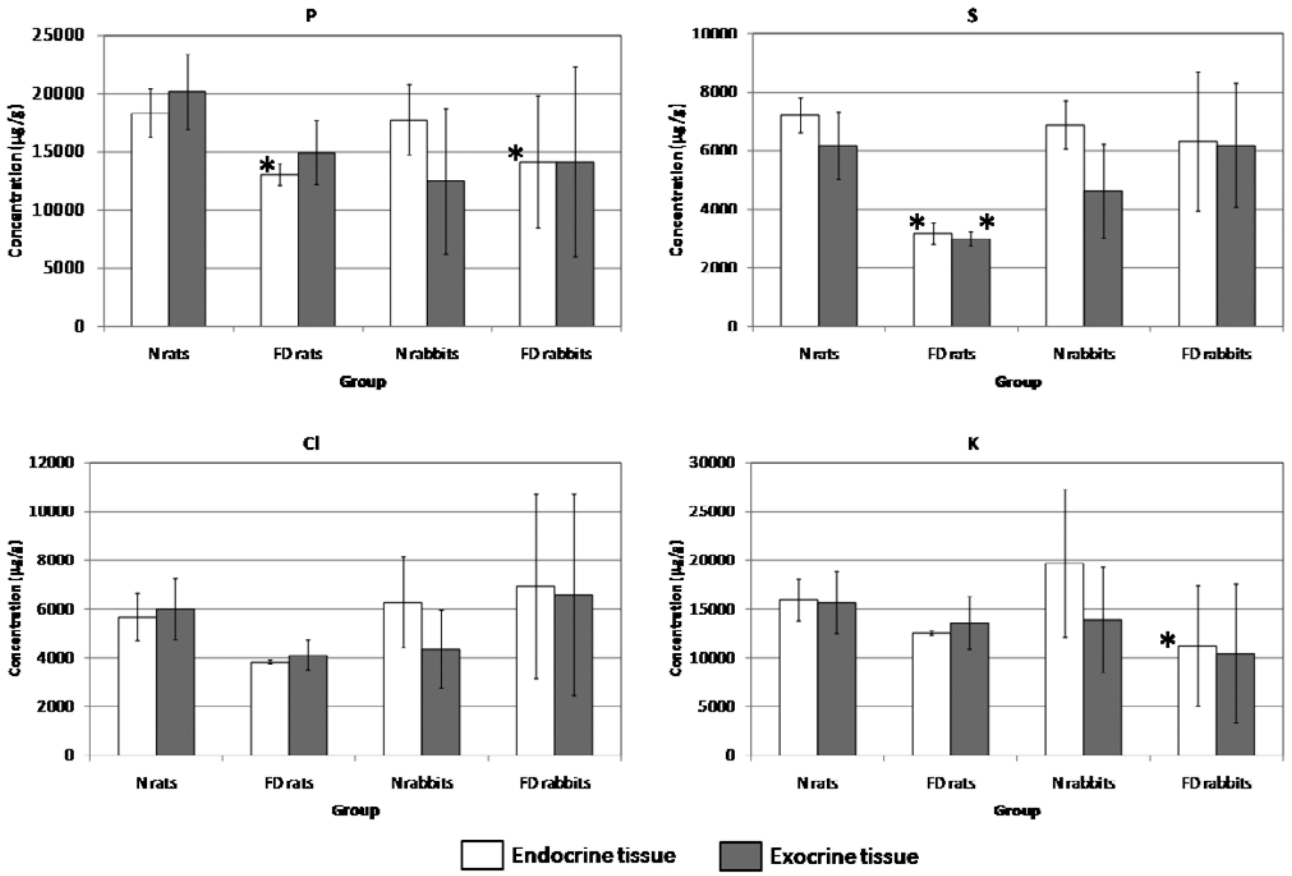

Figure 3. P, S, Cl, and K mean concentration and standard deviation of endocrine (white bar) and exocrine (gray bar) of rats and rabbits fed with standard diet (N rats and N rabbits), and with high fat diet (FD rats and FD rabbits). Significant differences ($p < 0.05$) to controls within species are indicated (*).

elements measured in the exocrine pancreas were significant ($p < 0.05$). The endocrine pancreas showed higher levels of Ca than exocrine pancreas in both species. Also, in both species the Zn distribution in the islets was heterogeneous and anticorrelated with Fe distribution as previously reported for rabbits (Ynsa et al., 2009).

As far as interspecies variability is concerned, it was observed that the concentrations of Zn in endocrine pancreas of rabbits were significantly higher than in rats (see Table 1). No other significant differences were encountered for elemental contents in endocrine and exocrine pancreas between rabbits and rats, as can be depicted from Table 1 and Figure 3.

Influence of High Fat Diet

High cholesterol and fat diet affected both rabbit and rat pancreas. Large deposits of fat were observed in endocrine and exocrine pancreas of rats and rabbits. The islet size decreased considerably in the animals fed with a high fat diet. In rats the fat deposits are massive, modifying typical organ morphological features. An H&E pancreas image of a rat under high fat diet (Fig. 4) was included for comparison purposes with Figure 1 (control rat pancreas). Islets could only be identified through Zn distribution maps as density contrast diminished due to fat deposition. In this pancreas region, the Zn distribution was homogenous and more diffuse than in animals with a normal diet.

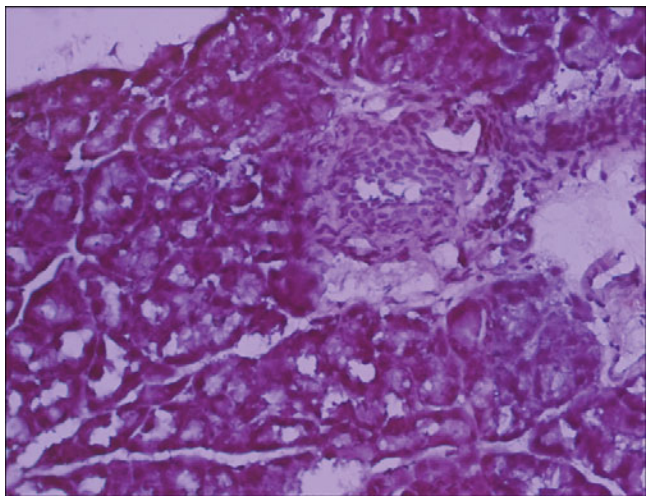

Figure 4. Pancreas of a rat under high fat diet. Sections stained with H&E.

In both rats and rabbits, the diet did not seem to affect the elemental levels of exocrine pancreas; however, it produced major alterations in the elemental contents of the islets. As can be inferred from Table 1, islets of rats and

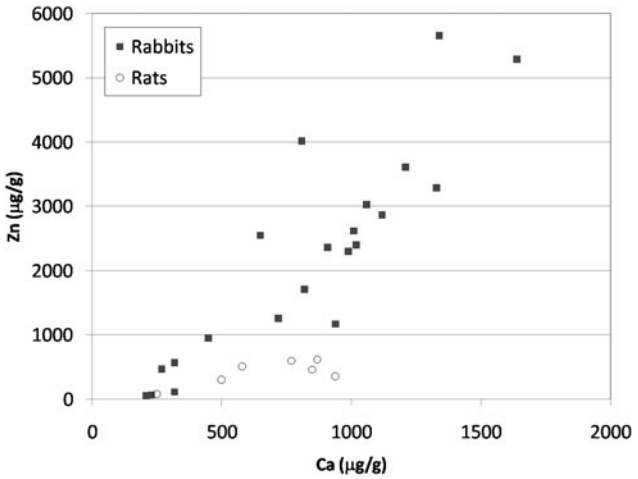

Figure 5. Association of Ca and Zn concentrations in endocrine pancreas. Gray squares correspond to both normal and FD rabbit pancreas and white circles correspond to both rat groups.

rabbits fed with a high fat diet showed a significant drop in Ca and Zn contents (rats, $p < 0.02$; rabbits, $p < 0.001$). These two elements were correlated in both rats and rabbits. Although the correlation coefficient was different in the two animal models (Fig. 5), the association was significant and independent of dietary condition.

Fe was also diminished in endocrine pancreas (Table 1), although the decrease was only significant in rabbits ($p = 0.003$). The P content of islets decreased in rats ($p = 0.02$) and rabbits ($p = 0.01$) under similar diet condition. In the islets of rabbits under a fat diet, a decrease of K was also observed ($p = 0.003$) whereas a significant decrease of S content ($p = 0.0003$) occurred in both exocrine and endocrine pancreas of rats fed with a fat diet (see Fig. 3).

Discussion

Rodents and rabbits have been extensively used in nutritional and metabolic studies and have helped to unravel many cellular mechanisms. In what concerns the effect of nutrient overload and/or deprivation in pancreatic functions, convergent results were not always obtained in *in vivo* and *in vitro* models (Tittle & Hume, 2008). In particular issues such as islet access of plasma lipids and minerals, and the effect of these nutrients on pancreatic islet function, are still under debate.

In the present study, two animal models were compared for pancreas morphological characteristics including the distributions of physiologically important elements. Both rats and rabbits were treated with diets containing cholesterol and FFA in relatively high levels (though not as extreme as in some studies, see Pinnick et al., 2008) to assess the sensitivity of nuclear microscopy imaging to identify tissue alterations. The pancreas of the two studied models, Spague Dawley rats and New Zealand White rabbits, showed morphological and biological differences of endocrine and exocrine pancreas, such as the number and size of Langerhans' islets, elemental distributions, and concentrations. Islets in rabbits are smaller than in rats and were not as easily distinguished in optical micrographs of H&E stained cryosections. These anatomical differences may be specific to the species. It appears that islet structure does not change in intact pancreas, but islet mass may expand during pathological conditions (Bock et al., 2003).

STIM images (mass density) could only clearly discriminate endocrine and exocrine pancreas in rats. The mass density images in a rabbit pancreas appear to be more uniformly distributed than in a rat pancreas, making the identification of endocrine and exocrine clusters more demanding. Islet secretory cells may contain void and packed secretory vesicles with constituents of relatively low density, such as insulin, glucagon, and calmodulin, and this may explain the density contrast observed between endocrine and exocrine tissues.

Exocrine and endocrine pancreas of rabbits and rats could also be distinguished by Ca, Fe, and Zn distributions. Islets had higher Ca, Fe, and Zn concentrations compared with exocrine tissues. The contents of Ca and Fe were similar in both species, but Sprague Dawley rats showed significantly lower levels of Zn in exocrine and endocrine pancreas than New Zealand White rabbits. The cause of this interspecies differentiation is not easily explained although it may reflect differences in metabolism. Nevertheless, the observed ratio of Zn concentrations between exocrine pancreas and islets was similar in normal rats and normal rabbits.

Information on elemental contents of pancreas in rodents and rabbits is scarce. Most of data refers to bulk analysis, which did not discriminate between variations in exocrine and endocrine pancreas. Moreover, concentrations reported in the literature varied by a factor of 100 (Tobia et al., 1998; Feng et al., 2001). An early application of nuclear microscopy (Juntti-Berggren et al., 1991) evaluated elemental contents of mice endocrine and exocrine pancreas, isolated manually by microdissection after cryosectioning. Although the measured concentration levels in mice differ from Spague-Dawley rats and rabbits analyzed in the present work, similar variations in elemental contents of endocrine and exocrine pancreas can be found. Lower contents of Ca, Fe, and Zn were described for exocrine pancreas relative to islets while the levels of P, S, Cl, and K were similar in both tissues (Juntti-Berggren et al., 1991). The determination of minerals such as Zn, Ca, and other nutrients in pancreas is still a demanding analytical task. The elemental concentrations in specific tissues and cells are usually measured by indirect methods. Intracellular Zn can be estimated from enzymatic inhibition constants, using radioactive tracers that may be measured by nuclear magnetic resonance or using fluorescent probes. These methods do not allow a precise quantification of the metal because these probes are not specific to single metals/cations and interferences of other ionic species are common (Beyersmann & Haase, 2001; Wagner et al., 2010). In this context, nuclear microscopy techniques offer outstanding value because trace-elemental concentrations can be directly evalu-

ated in the tissue at a cell-scale resolution with high quantitative accuracy. As elemental concentrations are assessed simultaneously, direct correlative analysis of the elements detected can be done. In addition chemical preparation and the use of contrasts are not required, drastically limiting elemental losses and mobilization and enabling analysis close to the *in vivo* condition.

In fact, direct association of density and elemental concentrations with pancreas morphology was possible in unstained cryosections of pancreas from both animal models studied. Consequently the association of Ca, Fe, and Zn high contents with islets can be related to their function in pancreas, either in rats or rabbits (Ynsa et al., 2009). Endocrine pancreas in both animal models also showed a negative correlation for Fe and Zn. The islets comprise clusters of endocrine cells interlaced by a dense network of specialized capillaries, which are lining the layers of endocrine cells and regulate islet blood flow (Ballian & Brunicardi, 2007). This cell-capillary arrangement is compatible with the anticorrelation of Fe and Zn observed for rats and rabbits, as most Zn should account for insulin pools in β-cells and Fe for blood constituents in capillaries. Also the high content of Ca in these regions may reflect the rise of cytosolic Ca requirements for secretion. Insulin release is initiated by the rise in cytosolic Ca^{2+} induced by adenosine triphosphate (ATP) production and the K-channel ATP-dependent triggering pathway (Nolan et al., 2006).

In animals fed with a high fat diet, alterations in the concentrations of electrolytes such as P and K (only in rats) and metals such as Ca, Fe, and Zn (both rats and rabbits), which are essential elements to maintain a functional pancreas, were shown. Therefore, the major decrease of Ca, Fe, and Zn concentrations in endocrine pancreas of animals, rats and rabbits, with excess fat suggests endocrine physiological changes that accompany the significant morphological alterations also observed. Especially in rat pancreas where massive fat deposits were observed, the mass density and S concentration decrease in endocrine and exocrine pancreas suggest that the number of cells diminished and lessen protein content, as lipids are less dense. It has been reported in the literature that the excess of saturated fatty acids alone or in combination with elevated glucose reduce insulin biosynthesis and secretion and induce β-cell apoptosis (Dandona et al., 2005; Nolan et al., 2006; Poitout & Robertson, 2008). The loss of endocrine cells of pancreas has been related to dyslipidemia and hyperglycemia associated pathologies and the mass of surviving β-cells has been indicated as a promising diagnostic for diabetes (Saudek et al., 2008). The rate of natural renewal from cell replication or putative adult stem cells available may not be enough to counteract cell loss and consequently the amount of functional endocrine cells decrease and islet mass likely decrease.

In addition to mass density reduction and Ca, Fe, and Zn decrease, the decline of P and K concentration in endocrine pancreas of the animals with excess fat also point out secretion impairment, as these elements are required in the metabolic pathways of hormone synthesis and release. The limited availability of P, observed in both rats and rabbits, can hamper mechanisms of protein synthesis, activation, or inhibition, among others, which are dependent on phosphorylation. The decrease of P may also reflect a broad decrease of islet metabolism. In turn, the decrease of K in the endocrine pancreas of rabbits (significant decrease) and rats (nonsignificant decrease) may influence membrane depolarization requirements for the secretion of the various hormones produced by islets cells, such as insulin, glucagon, and calmodulin.

Several animal studies (Ravier & Rutter, 2005; Wagner et al., 2010) have shown that excess of exogenous FFA and cholesterol suppressed glucose-stimulation insulin secretion that could be traced to changes in islets cells cytosolic Ca^{2+} and concurrent Zn^{2+} release. It is accepted (Gromada et al., 2007) that insulin release from granule pools involves the dissociation of Zn-insulin dimers complex and exocytosis open voltage-dependent Ca^{2+} channels activation while Zn is co-released. Therefore, Ca and Zn variations in islets seem to be linked. In this work a strong correlation was found for Ca and Zn concentrations in endocrine pancreas, which was independent of dietary fat intake, demonstrating that both elements are directly related to the same mechanism or pathway.

Conclusions

Nuclear microprobe techniques can clearly discriminate endocrine and exocrine pancreas in the two animal models studied, Spague Dawley rats and New Zealand White rabbits.

The results presented in this work show morphological and biological differences of endocrine and exocrine healthy pancreas of the two models, such as the size of the islets of Langerhans, elemental distributions, and concentrations.

The pancreas of animals fed with a high fat diet shown alterations in the concentrations of electrolytes such as P and K and metals such as Ca, Fe, and Zn, which are essential elements to maintain a functional pancreas. In addition, significant morphological alterations were also observed, which suggest physiological changes.

Future research directions should encompass metabolic parameters, detailed islet mass and islet cell proliferation rate, and apoptosis.

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