Regulatory Peptides 183 (2013) 62–68

Contents lists available at SciVerse ScienceDirect

Regulatory Peptides

journal homepage: www.elsevier.com/locate/regpep

What difference exists in the pancreas of mammals with sanguivorous diet? A morphological, stereological and immunohistochemical study of the pancreatic islets of the hematophagous bat *Diphylla ecaudata*

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ARTICLE INFO

Article history:
Received 12 November 2012
Received in revised form 26 December 2012
Accepted 3 March 2013
Available online 14 March 2013

Keywords:
Pancreas
Chiroptera
Endocrine cells
Histology

ABSTRACT

Diphylla ecaudata is a vampire bat that mainly feeds on the blood of birds. This highly specialized diet - hematophagy – is accompanied by a series of morphological changes in the gastro-entero-pancreatic system, since the distribution and relative proportions of different pancreatic endocrine cell types can vary between species due to different physiological conditions and eating habits. The aim of this study was to examine for the first time the pancreas of the vampire bat *D. ecaudata* using morphological, stereological and immunohistochemical techniques. The pancreas of the *D. ecaudata* has an exocrine acinar portion in which the highest concentration of pancreatic islets is scattered. These pancreatic islets have irregular size and a mean diameter of 56.94 μm. The total number of islets in the pancreas was 23,900, with a volumetric density of 4.1%. Insulin-immunoreactive (IR) cells were located in the central pancreatic islet region and had the largest density (54.8%). Glucagon-IR cells were located mainly in the peripheral mantle region (16.2%), along with somatostatin-IR (SS) cells (14.3%). Cells immunoreactive to insulin, glucagon and somatostatin were also observed to have spread in isolated places in the exocrine pancreas. In the connective tissue near the pancreatic ducts, a high concentration was identified of insulin-IR cells and a low concentration of glucagon-IR and somatostatin-IR cells. These results indicate that although the pancreas of *D. ecaudata* has morphological similarities with that of other mammals, it has a differentiated islet structure, because there were a large number of islets and different volumetric densities of α, β and δ cells.

1. Introduction

Although there are over 5400 species of mammals, only three of these are exclusively hematophagous, the bats of the Phyllostomidae family: *Desmodus rotundus*, *Diphylla ecaudata* and *Diaemus youngi*. These bats exist only in tropical regions, in Mexico, Central America, Peru and Brazil [40]. Besides their ecological importance, bats are important experimental animals, because they are more similar to humans in various morphological and biochemical aspects than are rats, which are commonly used in scientific experimentation for having a biliary vesicle, encapsulated pancreas and hepatic distribution of the PEPCK enzyme (associated with neoglucogenesis) similar to those of humans [37].

D. ecaudata (hairy-legged vampire bat) is the largest hematophagous bat species and preferably feeds on avian blood. It can ingest some 15–16 ml of blood per night, representing nearly half of its body weight [50,54]. The blood loss to birds can cause economic losses to poultry farmers by lowering the animal productivity. The blood diet of this bat is accompanied by a series of morphological modifications and adaptations in the digestive system. The stomach is very elongated, with a much larger absorption surface than in other bat species [41]. Despite the importance of this species, published studies are scarce due to the difficulty of breeding this bat in captivity and the small number of specimens typically found in wild colonies.

The pancreas is an organ that contains distinct sub-populations of cells: the exocrine cells, which secrete enzymes into the digestive tract; and the endocrine cells, which form islets and secrete their hormones into the bloodstream [45]. The structure of the pancreatic islets (islets of Langerhans) consists of different endocrine cells that secrete various hormones, which play a vital role in maintaining homeostasis [56]. The endocrine cell distribution within the pancreatic islets can vary both between species and under different energy-demand conditions [23].

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http://dx.doi.org/10.1016/j.regpep.2013.03.015

Studies of the endocrine cells in the stomach and gut of bats confirm these adaptations observed in species that have different types of diet [10,29,42].

The pancreas and its endocrine components have been described in studies of many different mammals, such as human neonates and infants [39], macaques [21], rodents [23,48,56], dogs [17], and pigs and cattle [9]. In the Chiroptera order, the endocrine cells of the pancreas have been described in the insectivorous bat *Myotis lucifugus* through ultrastructural analysis [2,34] and the fruit-eating bats *Artibeus lituratus* by immunohistochemical analysis of β cells [38] and *Rousettus aegyptiacus* by morphometric and immunohistochemical analysis [32].

Hematophagous bats have a diet poor in carbohydrates and rich in proteins (HP diet) since the blood's solid fraction is usually 93.1% protein [6]. But unlike carnivorous mammals, such as lions and tigers [12], and rodents [35] that have a HP diet, these bats are not able to live for more than 24–36 h without food due to the deficiency of the neoglucogenesis pathway [15].

Based on these metabolic differences and the fact that diet can lead to adaptations of the morpho-functional structure of the gastro-entero-pancreatic tract, the aim of the present work is to describe for the first time the pancreas of the vampire bat *D. ecaudata* through morphological, stereological and immunohistochemical analyses, to contribute to the knowledge of this species that has such a peculiar diet among mammals: blood.

2. Material and methods

The experimental procedures and animal care were approved by the institutional committee for animal use of Rio de Janeiro Federal Rural University, Seropédica, Brazil.

2.1. Capture of the bats and collection of the material

Six adult males were used; collected according to Brazilian law. The specimens were collected during the night with mist nets and hand nets, in Casa de Pedra cave in the state of Sergipe, Brazil. The bats were taken to Sergipe Federal University, where they were weighed and sacrificed with sodium thiopental at a dose of 100 mg/kg. Then median laparotomy was performed to remove the dorsal and ventral lobe of the pancreas. The volume of the pancreas (V [p]) was measured by the submersion method [43], in which the displacement of liquid (isotonic saline) attributable to V [p] was recorded by weight (W). Because isotonic saline's specific density (s) is 1.0048 g/ml, the respective volumes were obtained according to the formula V [p] (cm^3) = W (g)/s, or simply, V (cm^3) ≅ W (g) [53]. After being measured, each pancreas was fixed in freshly prepared Bouin's fluid for 6 h and preserved in a solution of alcohol 70 and sent to Rio de Janeiro Federal Rural University for histological processing by inclusion in paraffin. Five-micrometer thick serial slices were stained with hematoxylin-eosin (HE) and Gomori's trichrome [28] to observe the normal structure of the organ.

2.2. Immunofluorescence and immunohistochemistry

For immunofluorescence, antigen retrieval was accomplished using citrate buffer, pH 6.0, 60 °C for 20 min and blocked with ammonium chloride, 2% glycin, and phosphate buffer, pH 7.4 (PBS). Pancreatic sections were simultaneously incubated with rabbit anti-glucagon (A0565, Dako), guinea pig anti-insulin (A0564, Dako) and rabbit anti-human somatostatin (A0566, Dako). Primary antibodies were diluted to 1:50, 1:50 and 1:300 respectively in blocking buffer (PBS/1% BSA) and incubated overnight at 4 °C. Then the samples were incubated for 1 h at room temperature with fluorochrome-conjugated secondary antibodies: donkey anti-rabbit IgG-Alexa 488 and goat anti-guinea pig IgG-Alexa 546 (Invitrogen, Molecular Probes, Carlsbad, CA, USA), both diluted to 1:50 in PBS/1% BSA. After rinsing in PBS, the slides were mounted with DAPI nucleic acid stain and SlowFade Antifade (Invitrogen, Molecular Probes, Carlsbad, CA, USA). Double indirect immunofluorescence images were captured using a Zeiss model LSM 510 confocal laser scanning microscope (Meta, Germany). For immunohistochemistry, sections were incubated with anti-insulin (G 0785, Sigma-Aldrich) diluted to 1:1000, anti-glucagon (G 2654, Sigma-Aldrich) diluted to 1:2000 and anti-somatostatin (A0566, Dako) diluted to 1:300 and then amplified with a biotin–streptavidin complex (PK 6200; Vector). Insulin, glucagon and somatostatin were identified with 3,3′diaminobenzidine tetrachloride (H-2200, DAB, Vector) and sections were counterstained with Mayer hematoxylin. Digital images of the stained slices were obtained using an LC Evolution camera mounted on an Olympus BX51 microscope.

2.3. Pancreas morphometry

Five-micrometer thick sections were obtained from each pancreas and stained with hematoxylin and eosin. From the digital images of pancreatic tissue, the smallest and largest diameters of each islet were measured to calculate the mean islet diameter (Image-Pro Plus version 7.0, Media Cybernetics, Silver Spring, MD, USA). At least 100 islets were measured per animal.

2.4. Pancreas stereology

2.4.1. Islet number (N [islet])

The pancreatic islet number was estimated using a physical dissector–fractionator method [3]. Briefly, in a consecutive series of sections, starting with a random section and leaving an interval of 10 sections, the distance between look-up and look-down sections was 20 μm for each pair, as it represents about 1/3 of the islet diameter in these animals. Thus, islets seen in look-up in anterior sections but not the look-down sections were counted (Q_A^-), and the numerical density of islets (N_V) was estimated as: N_V [islet] = $Q_A^-/A_T * d$ ($1/mm^3$). The number of islets (N [islet]) was estimated as the product: V [p] * Nv [islet].

2.4.2. Islet volume density (Vv [islet]) and mass of islet (M [islet])

Vv [islet] was estimated by point-counting: the ratio of the number of points that hit the pancreatic islet (Pp) and the total number of test-points in a test-system made up of 36 test-points (P_T): Vv [islet] = Pp [islet]/P_T (%). Subsequently, the volume was obtained by multiplying the Vv [islet] by pancreatic mass [30].

2.4.3. α, β, δ cell volume density (Vv [α, β, δ cell])

Vv [α, β, δ cell] was estimated by image analysis using the density threshold selection tool applied to islets with insulin-positive areas. α, β, δ cell volume density was expressed as a percentage of the islet (Image-Pro Plus version 7.0, Media Cybernetics, Silver Spring, MD, USA) [31]. All the parameters analyzed were expressed as mean ± SD (standard deviation).

3. Results

3.1. Histology

The pancreas of *D. ecaudata* is located ventrally in the abdominal cavity near the duodenum and extends transversally to the stomach. The average mass of the pancreas was 0.1 g and the bats average body weight was 24.4 g.

The pancreas is covered by a thin capsule of loose connective tissue, which extends in the form of septums to the inside of the organ, subdividing the gland into visibly distinct lobules. These are formed of an exocrine part and endocrine part composed of pancreatic islets. These islets are easily identified and have irregular shape, and were slightly stained by eosin (Fig. 1A). The exocrine part of the pancreas is composed of acinar cells and a system of ducts, which starts with the formation of small center acinar cells that lead to intralobular ducts covered by a simple squamous or cubic epithelium. The intralobular ducts

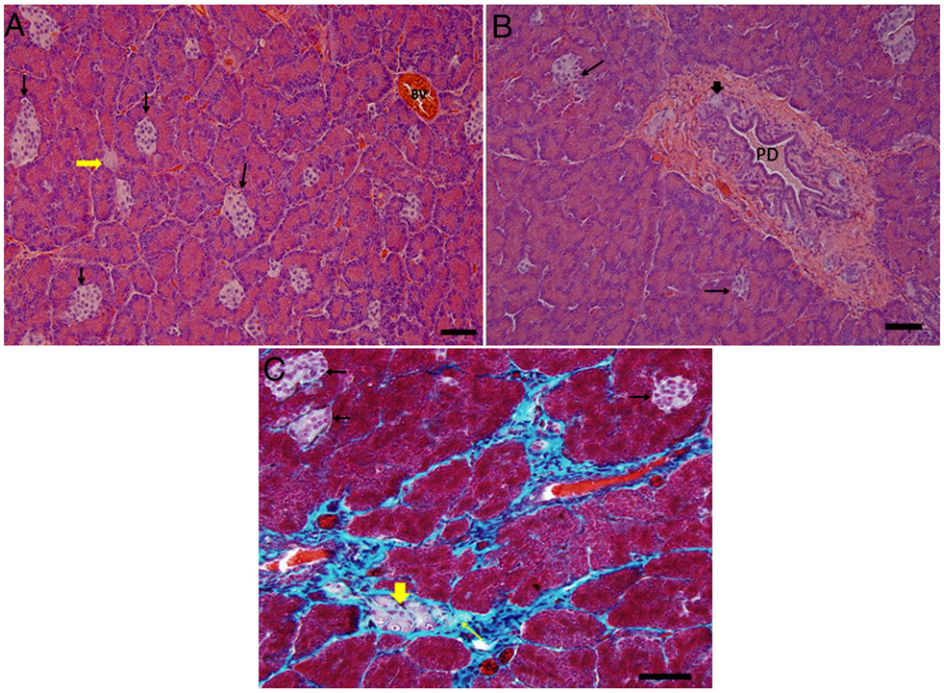

Fig. 1. Photomicrographs of the pancreas of *D. ecaudata*. A. Note the high concentration of pancreatic islets scattered throughout the exocrine pancreas. Blood vessel (BV) and ganglion neuron perikarya (yellow arrow). HE. B. Pancreatic duct with small mucous glands (thick arrow). HE. C. Ganglion among connective tissue (yellow arrow). Gomori's trichrome. Pancreatic islets (black arrows). Scale bar = 50 μm.

converge to form the interlobular ducts, which are covered with a simple cubic (Fig. 4A) or columnar epithelium. The interlobular ducts in turn empty into the main pancreatic duct, which is lined with a simple columnar epithelium enclosed by connective tissue containing small mucous glands that open into larger ducts (Fig. 1B). The presence of ganglia and nerve fibers was also observed (Fig. 1C).

3.2. Pancreas morphometry

The average diameter of the pancreatic islets was 56.94 μm ± 16.80.

3.3. Pancreas stereology

The average number of pancreatic islets (N [islets]) observed was 23,900 ± 9770.54, the volumetric density of the islets (Vv [islets]) was 4.1% and the mass of the islets (M [islets]), i.e., the mass of the endocrine tissue in the total pancreatic tissue, was 0.48 mg.

3.4. Immunofluorescence and immunohistochemistry

The insulin-immunoreactive (IR) cells are located in the central part (Fig. 2A, B) and the glucagon-IR cells preferentially in the peripheral part, but these cells were also found in the center of the islets with the presence of a cytoplasmic process (Fig. 2C, D). The somatostatin-IR cells are also located in the periphery (Fig. 2E, F).

Regarding the volume density of the endocrine cells, this was greatest in the β cells (54.8% ± 10.1%), followed by the α cells (16.2% ± 5.1%) and δ cells (14.3% ± 4.3%) (Fig. 3).

In the connective tissue itself near the pancreatic ducts, high concentrations were identified of insulin-IR cells (Fig. 4B, C) and low concentrations of glucagon-IR and somatostatin-IR cells (Fig. 4D).

The immunofluorescence confirmed the predominance of insulin-IR cells in the center of the islets, with glucagon-IR and somatostatin-IR cells located in their periphery (Fig. 5A–D). IR cells to insulin, glucagon and somatostatin were also observed in isolation in the exocrine pancreas, that is, outside the pancreatic islets, in all the specimens examined (Fig. 5B, C).

4. Discussion

The Chiroptera family stands out from other mammal families for its great trophic diversity. This trophic diversity includes hematophagy, a diet that consists predominantly of protein with a low quantity of glucose. The ability to obtain adequate energy from such a diet is partly due to morpho-physiological modifications and adaptations in the gastrointestinal tube and the endocrine portion of the pancreas of these animals [22]. These adaptations of the pancreas have been reported for fruit-eating bats, such as *R. aegyptiacus*, in which the endocrine pancreas represents 9.1% of the total pancreas volume, far more than in some other mammals [32], and *A. lituratus*, in which the presence of an apparently large β-cell distribution observed is an adaptation that guarantees the proper control of glucose homeostasis in this species, which is constantly submitted to high influx of glucose due to its carbohydrate-rich diet [38].

The pancreas of *D. ecaudata* was histologically similar to that of the fruit-eating bats *R. aegyptiacus* [32] and *A. lituratus* [38], in which richly vascularized and irregularly shaped islets were visually observed separate from the exocrine pancreas.

Studies of the quantification and morphometry of the pancreatic islets of vertebrates have been performed for different purposes. In a study aiming at pancreas transplantation, it was found that the diameters of the pancreatic islets in humans and dogs are similar, with average of 150 μm [52]. Other studies have related the size of the islets with the quantity of secretions of insulin and glucagon in the pancreas of obese rats, defining large islets (>0.45 mm) and small islets (<0.12 mm), the former producing more insulin than the latter [19]. Another study measured the diameter of the islets in Wistar rats and Bonnet macaques (*Macaca radiata*), with the rat islets

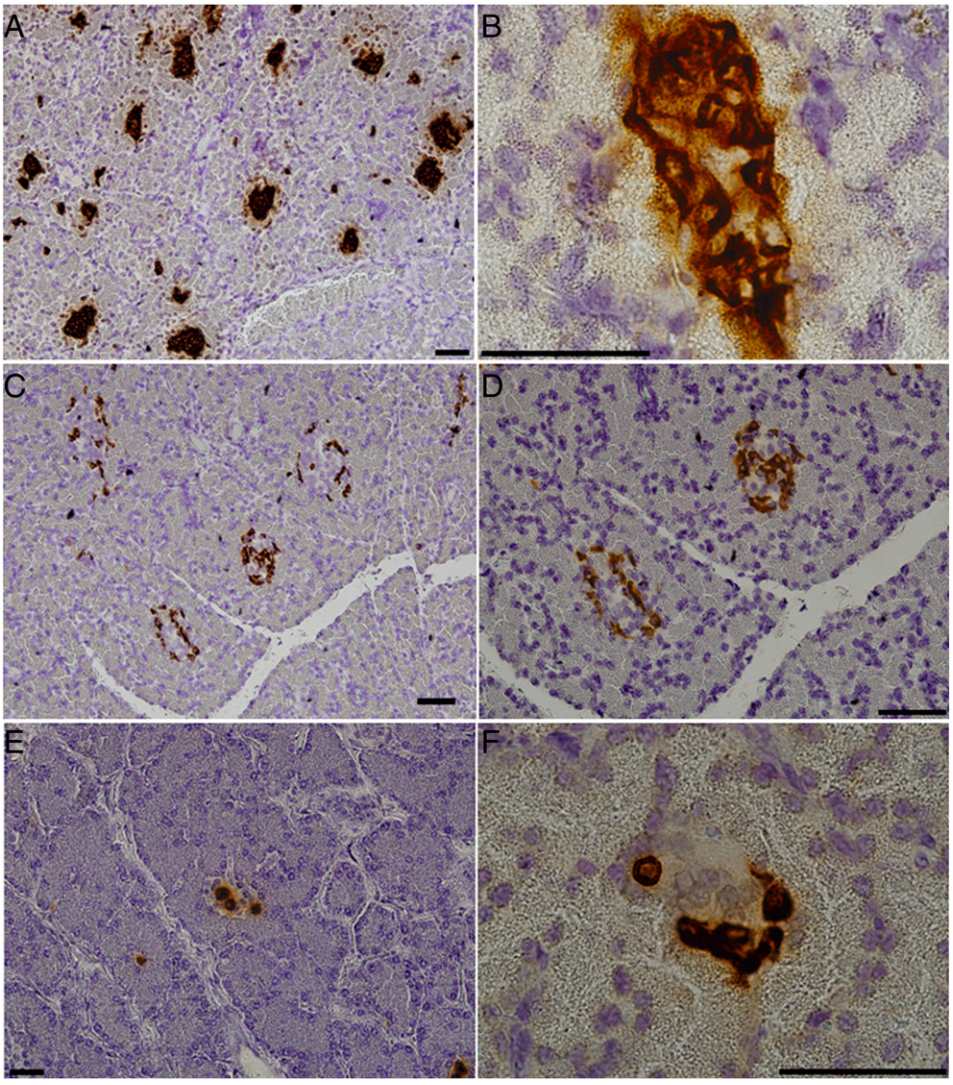

Fig. 2. Photomicrographs of the pancreas of *D. ecaudata*. A, B. insulin-IR cells with ample extension in the pancreas. C. Glucagon-IR cells. D. Glucagon-IR cells located in the peripheral and central part of the pancreatic islet. E. Somatostatin-IR cells. F. Somatostatin-IR cells in the peripheral pancreatic islet region. Scale bar = 50 μm.

averaging 201 μm against 230 μm in the macaques, demonstrating that the animal's weight and size (200–250 g for rats and 3–5 kg for macaques) are not related to the size of the pancreatic islets [11,47]. In the present study, the size in *D. ecaudata* was 56.94 μm. This measurement has not been performed in bats previously, so no comparisons with other bat species are possible.

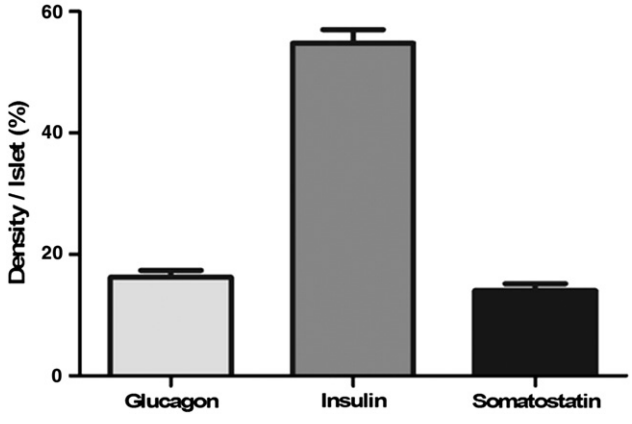

Fig. 3. The volumetric densities of insulin-IR cells, glucagon-IR cells and somatostatin-IR cells in *D. ecaudata*.

The quantitative evaluation of the structure of an organ and of its cellular components, based on stereological examination of tissue samples, is a powerful tool in biomedical investigations. Stereology is based on geometric and statistical considerations and permits obtaining impartial estimates of the volumes, surfaces, sizes and numbers of the structures of interest [49].

In this study we used this method to quantify the total number of pancreatic islets for the first time in bats, finding that the vampire bat *D. ecaudata* has an average N [islets] of 23,900. This number is greater than that observed in various lines of rats, which despite having a body mass (±25 g) and pancreatic tissue mass (±1–2 g) similar to that of vampire bats, have N [islets] ranging from 1000 to 3500 [3]. These authors suggested that specific genes regulate the variation of the size and structure (number of islets, mass of islets, mass of α and β cells) of the endocrine pancreas. In a study of the large Japanese filed mouse (*Apodemus speciosus*), a larger number of islets was observed in the dorsal portion of the pancreas than in the ventral portion [56].

The volume density of the islets (Vv) of *D. ecaudata* was 4.1%, lower than that found in the fruit-eating bat *R. aegyptiacus*, which was 9.1% [32], and in human infants (6–7%) and neonates (15%)

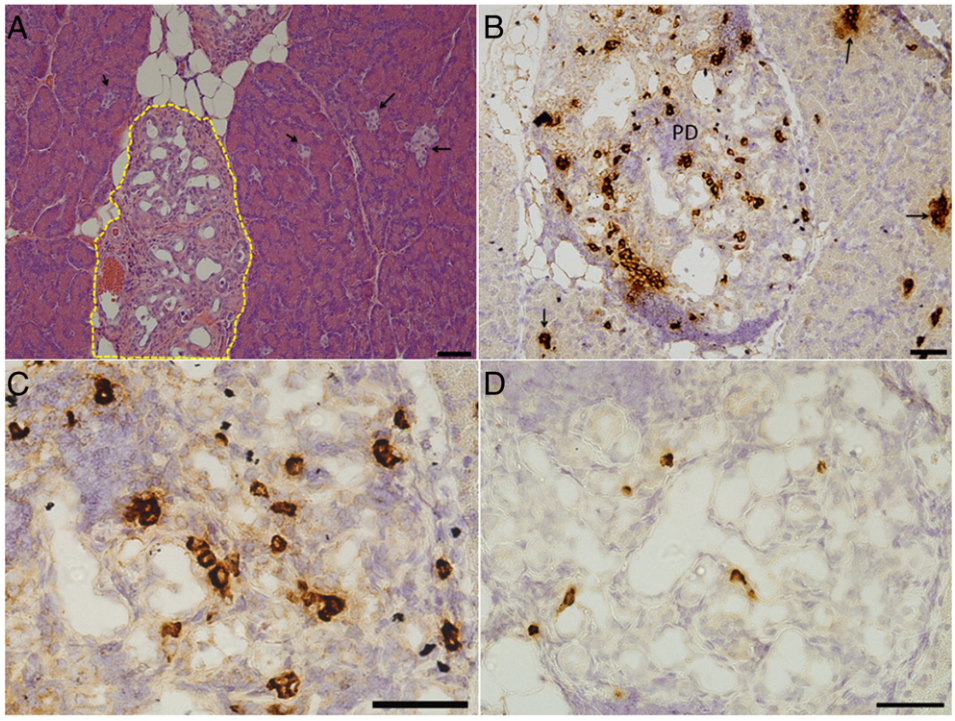

Fig. 4. The interlobular ducts in the hematophagous bat *D. ecaudata*. A. The interlobular ducts with a simple cubic epithelium (yellow circle). HE. B, C. Insulin-IR cells. D. Somatostatin-IR cells in the pancreatic duct (PD). Pancreatic islets (black arrows). Scale bar = 50 μm.

[39]. Despite this lower percentage in the present study, the figure is still higher than that observed in adult humans, where the Vv is 2–3% [39], and near that observed in adult rats, where the Vv is around 4% [14], and in cattle, where it is 5% [4]. The higher volumetric density of the endocrine tissue in fruit-eating bats can be explained as a morphological adaptation necessary to control the high inflows of glucose during feeding [38], in contrast to hematophagous bats, whose diets do not contain carbohydrates.

In the present investigation, the mass of the islets (M [islets]), i.e., the mass of the endocrine tissue occupied in the pancreas, was 0.48 mg for a pancreas weight of about 100 mg. In humans this mass has been found to vary according to age and weight: in neonates weighing 311 mg in 3 g of pancreatic tissue; in children, 440 mg in 8 g of pancreatic tissue; and in adults, 1494 mg in 70 g of pancreatic tissue [39].

The pancreatic islets are small organs located in the pancreas that are crucial for glucose homeostasis. Islets typically consist of four types of secretory endocrine cells, namely, insulin (β) cells, glucagon (α) cells, somatostatin (δ) cells, and pancreatic polypeptide-producing (PP) cells [7]. The location pattern of the endocrine cells in the pancreatic islets varies among species and their distribution in the islets in phylogenetically distinct species may have some functional significance. Another factor determining the number and distribution of these endocrine cells is the embryological origin of the pancreatic lobes [44]. In rodents there is a concentrated distribution of these cells in the islets, such that the β cells are mainly located in the center and the other cell types are in the periphery [8,25–27]. However, in humans this distribution is open to discussion, with one study having considered this arrangement to be present, but less evident [45] and another study finding a random arrangement [7].

The β cells, secreting insulin, are the main ones responsible for maintaining normal blood glucose levels (euglycemia), both in neonates and adults, with their sub-population being dynamic according to the compensatory changes in the organism [5]. In *D. ecaudata*, the β cells had a similar distribution pattern to that observed in the fruit-eating bat *R. aegyptiacus* [32] and rodents [1,7], where the β cells are located throughout the islets, but especially in the core. In the bat *A. lituratus*, the β cells seem to be located mainly in the periphery of the islets [38].

The β cells are the predominant type in the pancreatic islets, found in percentages between 60 and 70% in humans, dog, and rodents [33,55]. In *D. ecaudata*, these cells presented a volume density of 54.8%, a result near that observed in the bat *R. aegyptiacus* [32], where this cell type accounted for 47.4% of the endocrine cells.

The α cells of the pancreas synthesize glucagon and are responsible for controlling the levels of glucose in the blood. They were found in the periphery in *D. Ecaudata*, but they also could be observed irradiating from the periphery to the core. Peculiar distributions of cells have been previously reported in specific disease states [16,46,51] as well as certain rodents such as BALB and hairless mice [26,27], macaques [21] and horses [20], where the pancreatic islet contained a central mass of glucagon cells surrounded by insulin cells. The cyto-architectural distribution of endocrine cells within the islets is considered to be important in their functional interactions and may reflect paracrine functions and cell–cell interactions of the pancreatic endocrine cells [36].

There are variations among species regarding the frequency of this cell type. In *R. aegyptiacus* it represents 28.5% [32] while in *D. ecaudata* it accounted for 16.2% of the endocrine cells present in the islets, with a greater differentiation in frequency compared to the β cells.

The δ cells play an important role in the paracrine control of the development of α and β cells and also in the maturation of these cells [39]. They normally have low frequency in the pancreatic islets. In the present study, these cells were mainly found in the periphery of the islets, a pattern also observed in *R. aegyptiacus* [32]. In *R. aegyptiacus*, these cells accounted for 7.8% of the endocrine cells [32] while in the vampire bat *D. ecaudata* this figure was 14.3%, also showing the notable distinction in density when comparing this bat against others with different diets. This indicates a possible adaptation of the species to the feeding habit. However, other studies demonstrate that changes in all cell type concentrations and locations also can occur due to changes in energy requirements or in response to various physiological stress and pathological states [23].

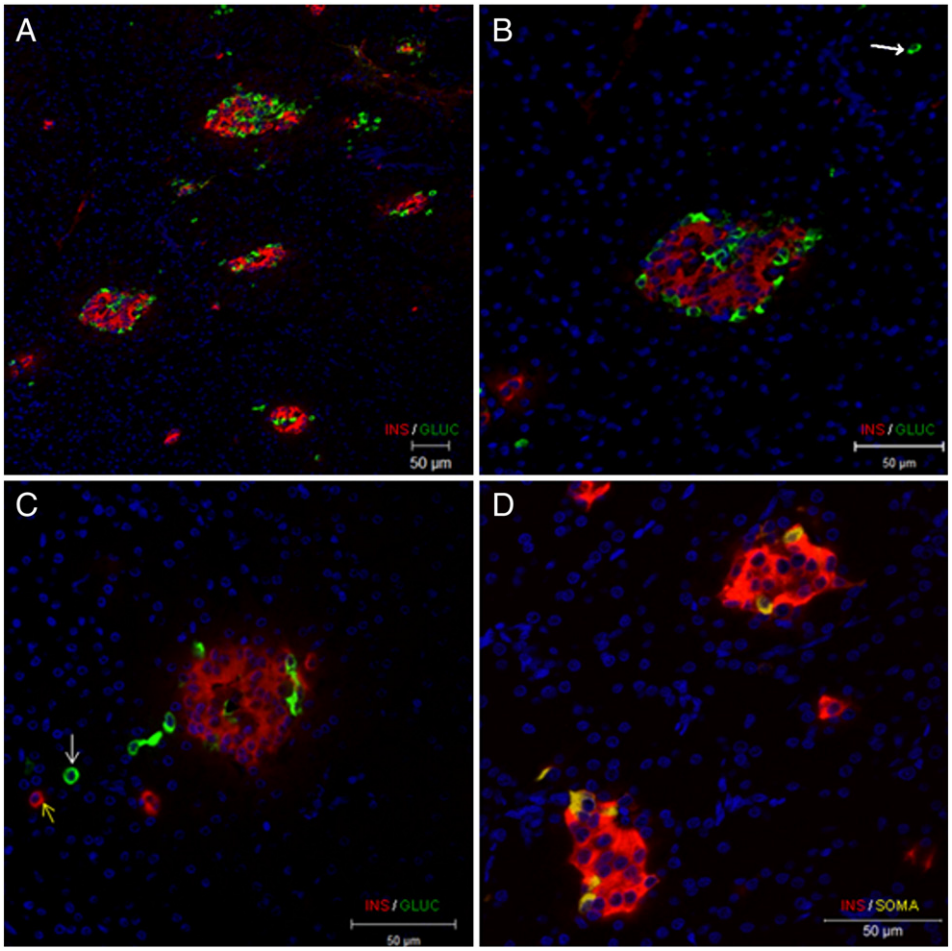

Fig. 5. Photomicrographs obtained by confocal microscopy after immunofluorescence of the pancreas of *D. ecaudata*. A. Note the high numerical index of pancreatic islets with insulin (red) and glucagon cells (yellow). B. insulin-IR cells in the center of the islets and glucagon-IR located in is periphery. Observed a glucagon-IR outside the pancreatic islets (white arrow). C. IR cells to insulin (yellow arrow) and glucagon (white arrow) scattered in exocrine pancreas. D. Somatostatin-IR cells and insulin-IR cells in the periphery and center of the islet, respectively.

In *D. ecaudata* we observed the presence of a cytoplasmic process in the α cells, which was also identified in the cells secreting somatostatin and polypeptide in the pancreas of the bat *R. aegyptiacus* [32]. This demonstrates a possible paracrine activity of these cells on the secretion of other hormones.

In the present investigation, the three cell types studied were found dispersed in the exocrine portion of the pancreas, similar to the pattern observed in the BALB/c mouse [26]. These endocrine cells were also observed around the pancreatic ducts or epithelium of the ducts, similar to the pattern reported in previous studies in dogs [33], horses [13], goats [24] and rodents [27]. The quantity of these cells in the duct region can be related to the age of the animals, being more frequent in fetuses and neonates [18] and young animals [19]. This location of endocrine cells in the region of the pancreatic ducts is generally found in higher mammals and is considered species-dependent [26].

In conclusion, the results indicate that although the pancreas of *D. ecaudata* has morphological similarities with that of other mammals, such as well-differentiated division between an endocrine and exocrine pancreas, well-defined islets and a larger number of insulin-IR cells, the pancreas of this vampire bat has a different islet structure (high numerical index) and different volumetric densities of α, β and δ cells. These characteristics might reflect evolutionary adaptations to different dietary habits (hematophagy) or other environmental constraints. Further studies are necessary to shed more light on the phylogenetic relations of this order and to propose hypotheses about the relations between eating habits and the morphology of the digestive tract of bats in particular and mammals in general.

Acknowledgments

The author thank Ilza Lucas Coelho Meirelles for her excellent technical assistance and Professor Carlos Alberto Mandarim-de-Lacerda for his assistance with experiments and the Office for Improvement of University Personnel (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior — CAPES) for the financial support.

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