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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*

Clarice Machado-Santos [a,*], Júlio César Fraulob Aquino [b], Jefferson Simanas Mikalauka [c], Marcelo Abidu-Figueiredo [d], Rosa Maria Marcos Mendes [d], Armando Sales [d]

[a] *Department of Biomorphology, Federal University of Bahia, Av. Reitor Miguel Calmon s/n, 40110-903, Salvador, BA, Brazil*
[b] *Laboratory of Morphometry, Metabolism & Cardiovascular Disease, Biomedical Center, Institute of Biology, State University of Rio de Janeiro, Av. 28 de Setembro 87 fds, 20551-030 Rio de Janeiro, RJ, Brazil*
[c] *Animal Biology Program, Rio de Janeiro Federal Rural University (UFRRJ), Br 465 km7 s/n, 23890-000, Seropédica, RJ, Brazil*
[d] *Animal Biology Department, Department of Animal Biology, UFRRJ, Seropédica, RJ, Brazil*

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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].

* Corresponding author. Tel.: +55 71 32838888; fax: +55 71 32388894.
E-mail address: clahisto@gmail.com (C. Machado-Santos).

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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