Microscopic study of the thymus of Guiana dolphin and Humpback whale

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Studies regarding thymus in cetaceans are scarce, with no report to the species occurring in Brazil. Due to an increasing concern about marine mammals' immunological status and how it is affected by the environment, this study aimed to describe histological characteristics of the thymus in two cetacean species with high occurrence in the Brazilian coast: the Odontoceti Guiana dolphin (*Sotalia guianensis*) and the Mysticeti Humpback whale (*Megaptera novaeangliae*). Guiana dolphins (3 calves, 1 young and 2 adults) and Humpback whales (2 calves, 1 young and 1 adult) were used and thymus samples were analyzed by light, scanning and transmission electron microscopy (SEM and TEM, respectively). The thymus was a lobulated lymphoid organ, anatomically located in the anterior superior mediastinum, in front of the heart. It was enclosed by a connective tissue capsule from which several connective tissue trabeculae arise and under which there was a cortex with an extensive network of interconnecting spaces. These spaces resembled a bee hive and were colonized by immature T-lymphocyte. There were several blood vessels in the capsule and trabeculae. The trabeculae extend into the interior of the organ, consisted of a dark-staining outer cortex and a light-staining inner medulla. The cortex contained densely packed lymphocytes, without the formation of lymphatic nodules. The medulla had fewer lymphocytes and more epithelial reticular cells that form the thymic (Hassall's) corpuscles. These corpuscles were round/oval structures consisting of spherical aggregations (whorls) of flattened epithelial cells and exhibiting calcification or degeneration centers. No adipose tissue replacing the thymic tissue was found in young animals. There were no differences between the species for any histological features. However, with regard to age, Hassall's corpuscles were more prominent in young animals, as the result of a decline in the lymphocyte production with age. However, this structure function remains uncertain. Thymus from cetaceans was similar to the observed in domestic mammals with involution related to age. The data presented in this study may contribute to a better understanding of the physiological processes related to the immunity of Odontoceti and Mysticeti during their growth.

Keywords: cetaceans; histology; immune system

1. Introduction

The cetacean thymus has a typical mammalian organization, including cortex, medulla and Hassall's corpuscles [1, 2]. This organ has been observed in several cetaceans' species such as the Bottlenose dolphin (*Tursiops truncatus*) and the Amazon River dolphin (*Inia geoffrensis*) [3]. However, little information on these typical features is known, especially regarding animals' age and gender, with most reports of thymic structure based on young animals [4].

The thymus involutes as it loses lymphocytes progressively with increasing age. Thymus lobules may display fat infiltration and/or involution, this due to Hassall's corpuscles degeneration or condensation of the thymus epithelial reticulum. Cysts development can also be observed, in the thymic tissue, completely replacing it in *Tursiops truncatus* [2]. However, the thymus can be easily visualized macroscopically in adult individuals, with some remnants being observed in senile Harbor porpoises and bottlenose dolphins. Such findings suggest that thymic involution is a slow and progressive age-related process in cetaceans [4, 5].

Studies regarding thymus in cetaceans are scarce, with no report to the species occurring in Brazil. This, allied to the fact that there is an increasing concern about marine mammals' immunological status and how it is affected by the environment, this study aimed to describe histological characteristics of the thymus in two cetacean coastal species with high occurrence and more susceptible to anthropic impacts in the Brazilian coast: the Odontoceti Guiana dolphin (*Sotalia guianensis*) and the Mysticeti Humpback whale (*Megaptera novaeangliae*).

2. Material and methods

Ten cetaceans, 6 Guiana dolphins (3 calves, 1 young and 2 adults) and 4 Humpback whales (2 calves, 1 young and 1 adult), stranded on the Northeast coast of Brazil (Sisbio Permit 37369-1, Animal Bioethics Protocol 2571/2012) were used in this study. For necropsy, animals were positioned in lateral decubitus and the initial incision was made on their left flank [6], allowing easy access to their thoracic cavity. Thymus was identified, photographed and tissue samples were washed, cut and fixed in 10% formalin solution for microscopy analysis. All samples were obtained in the research institutions Fundação Mamíferos Aquáticos – FMA (Sergipe, Brazil), Associação de Pesquisa e Preservação de Ecossistemas Aquáticos – AQUASIS (Ceará, Brazil) and Instituto Biota de Conservação – BIOTA (Alagoas, Brazil).

For light microscopy, samples were rinsed in tap water, dehydrated, diaphanized and embedded in histological paraffin (Paraplast®; Sigma-Aldrich®). Sections (6μm) were stained with Hematoxylin-Eosin and Masson's Trichrome and images were captured and analyzed using a light microscope (Nikon Eclipse E-800).

For scanning electron microscopy, samples fixed in 10% formalin were rinsed in distilled water and dehydrated in increasing series of ethanol with agitation. Then, they were critical-point dried, mounted on metal stubs using carbon adhesive, and coated with gold by sputting (Balzers-040 SDC). Samples were then examined in a scanning electron microscope (LEO 435 VP).

For transmission electron microscopy, samples were fixed in modified Karnovsky solution (2.5% of glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer solution) and post-fixed in 1% osmium tetroxide solution with 0.1M sodium phosphate buffer solution at 4°C for 2 h. Then, samples were dehydrated and embedded in Spurr resin and propylene oxide (1:1). Thereafter, this solution was replaced by pure resin and samples were placed in rubber molds in an oven for polymerization. After this procedure, 1-3 µm sections were obtained using an Ultra Cut® microtome (Reichert), stained with Toluidine blue, and examined under a light microscope (Nikon Eclipse E-800) to locate areas of interest and capture their images.

3. Results

The thymus was a lobulated lymphoid organ, anatomically located in the anterior superior mediastinum, in front of the heart as shown in Fig. 1A. Thymus was found only in calves and young animals and all three adults evaluated did not have any sigh of thymic tissue, with only a small quantity of adipose tissue near their hearts where the organ should be located. There were no differences between the species for any thymic histological features. The organ was surrounded by a connective tissue capsule, from which connective tissue trabeculae arise (Fig. 1B).

The trabeculae extend into the interior of the organ and subdivide the thymus parenchyma into an extensive network of interconnecting spaces (Fig. 1B), resembling a bee hive as presented in Figure 1C, colonized by epithelial cells and immature T-lymphocytes ("thymocytes").

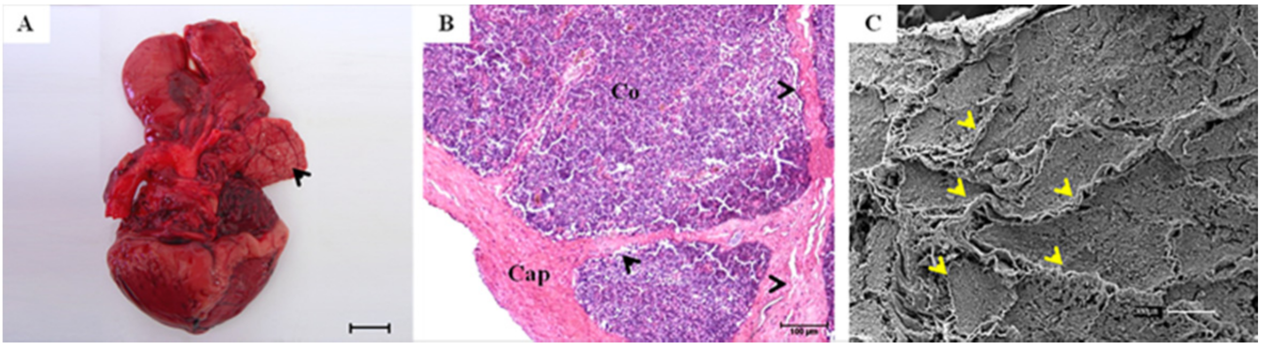

Fig. 1 Thymus of a Humpback whale calf (A) and a young Guiana dolphin (B, C). In A observe the thymus (arrowhead) located in front of the heart. Bar: 2cm. In B the capsule (Cap) surrounding the thymus gave rise to a trabecula that entered the parenchyma (arrowheads) and subdivided the cortex (Co) into several small interconnecting spaces. Hematoxylin-Eosin. In C note the extensive network formed by trabeculae (arrowheads), resembling a bee-hive. Scanning electron microphotograph.

Thymocytes had a round or oval nucleus and a not abundant cytoplasm (Fig. 2A). In Fig. 2B, both trabeculae and connective tissue capsule were highly irrigated and blood vessels passed into the thymus gland via these two structures.

The basic structural thymic unit is the lobule, being each one consisted of two distinct compartments: an outer cortex (dark-staining) and an inner medulla (light-staining) (Fig. 2C). The cortex contained densely packed lymphocytes, without the formation of lymphatic nodules. The medulla had fewer lymphocytes and more epithelial reticular cells, including the thymic (Hassall's) corpuscles.

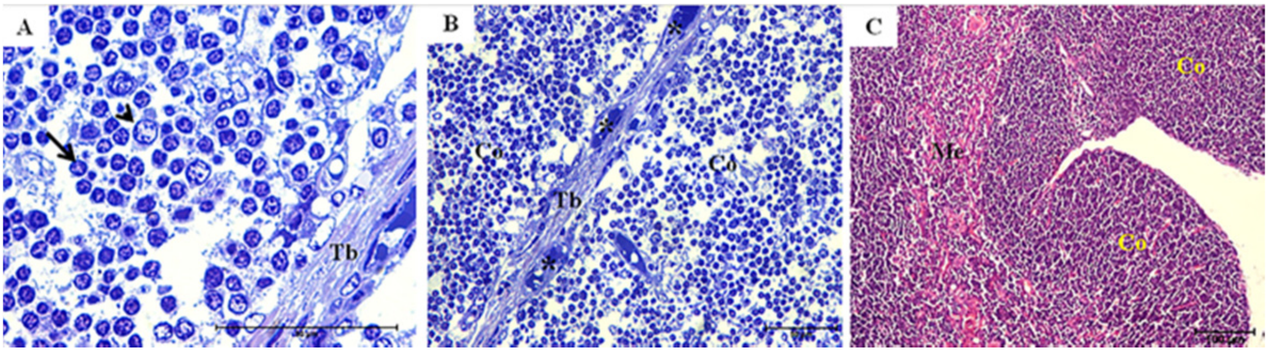

Fig. 2 Photomicrographs of thymus from Guiana dolphin calves (A, B) and a young Humpback whale (C). In A thymocytes (arrow) had round-shaped nuclei. Few lymphoblasts (arrowhead) were observed. Toluidine blue (TB). In B a trabecula containing several blood vessels (*), divided the thymic cortex (Co). TB. In C a dark-staining cortex (Co) surrounded a light-staining medulla (Me). Hematoxylin-Eosin.

Hassal's corpuscles (HC) were epithelial cells restricted to the thymic medulla, being the most easily identifiable microscopic structure of the thymus, as seen in Fig. 3. These corpuscles had a round/oval shape and consisted of spherical aggregations (whorls) of flattened epithelial cells, exhibiting calcification or degeneration centers. An active thymus in young animals presented a plumb HC, being smaller in calves (Fig. 3A, 3B and 3C) and more prominent in young animals (Fig. 3D, 3E and 3F). However, the functional significance of these corpuscles remains unknown. No adipose tissue replacing the thymic tissue was found in young animals.

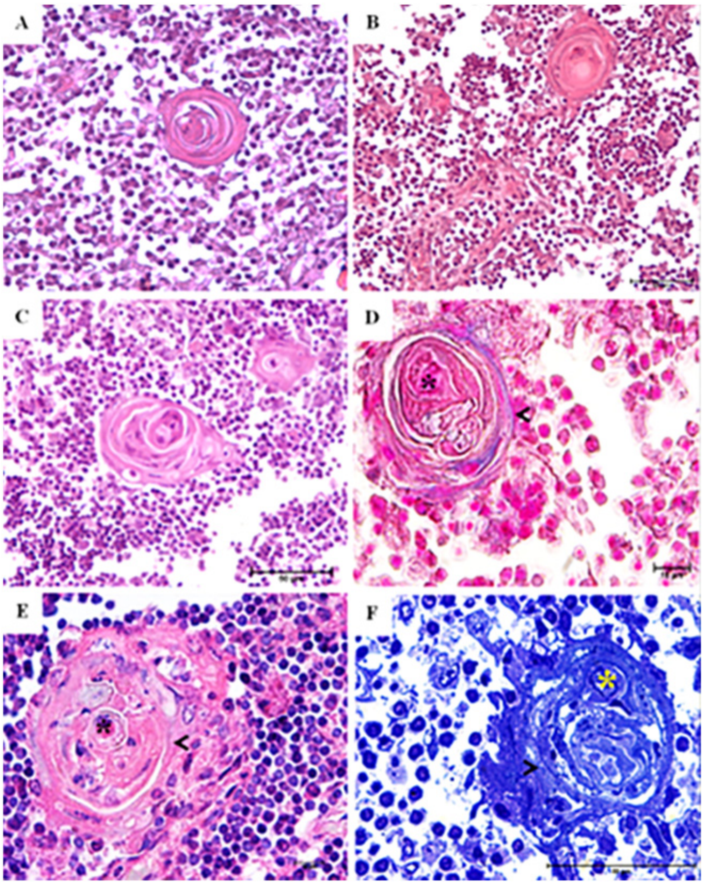

Fig. 3 Photomicrographs of Hassal's corpuscles from calves (A-C) and young (D-F) animals' thymus. A, B, E. Guiana dolphin; C, D, F. Humpback whale. In A, B and C observe the small Hassal's corpuscles in calves' thymus, surrounded by thymocytes, in comparison to D, E and F, where the corpuscles are bigger and more prominent in young animals, being formed by spherical aggregations surrounding a distinct nucleus. Hematoxylin-Eosin (A-C, E). Masson's Trichrome (D). Toluidine blue (F).

4. Discussion

The thymus remains one of the least understood lymphoid organs. Especially regarding the thymopoiesis, a complex and highly dynamic process, which involve tissue interactions between thymus-derived cells (T-cells) and the thymic microenvironment [7]. Despite the fact that it contains only two main cells, this organ has an important immunosurveillance role in both young and adult animals.

Some researchers evaluated the thymus function through its removal in rodents and reported that surgical effects differ, being directly dependent on animals' age. Mice thymectomized within a day of birth became susceptible to several infections and had growth deficiencies while in adults thymectomy does not have a immediate obvious effect. Thymectomy implies that neonatal thymus is the source of most blood lymphocytes mainly responsible for mounting cell-mediated immune responses [8].

Cunningham et *al.* [9] also corroborate with these data, reporting that in sheep naive T-cells constantly enter the peripheral T cell pool at the same rate throughout fetal, neonatal and adult life, showing that the thymus has an important and continuous immunological role throughout pre and post-natal life. Such evaluation was not possible to be performed in our study, as well as for most marine mammal species, due to several factors which make impossible to collect this type of data, such as a wide variation in age of the animals stranded.

The histology of the thymus gland varies with the age of the individual. The thymus gland attains its greatest development shortly after birth. By the time of puberty, thymus glands begin to involute or show signs of gradual regression and degeneration. As a consequence, lymphocyte production declines, and Hassall's corpuscles (HC) become more prominent [10,11]. In our study, calves had a smaller and more discrete HC, while older animals had their HC with more spherical aggregations (whorls), exhibiting calcification or degeneration centers

In our study, no remnant of the thymus was found in adult animals, which was completely replaced by adipose tissue. This finding is not consistent to the observed in bottlenose dolphin (*Tursiops truncatus*) [12] and Harbour porpoises (*Phocoena phocoena*) [5]. Thymic involution has been reported for several species [13,14,4]. In bottlenose dolphins, thymus involution was observed, together with the formation of epithelial cysts in older animals [2]. This cystic formation was reported in Harbour porpoise [5], suggesting that cystic formation is not related to animals' habitat or live habits. No thymic tissue were observed in our older animals, therefore cysts were not possible to be observed in our study.

Involution of the thymus and certain other lymphoid tissues has been documented in Harbor porpoises, humans, dogs, and cattle, but the rate at which it occurs has not been defined [5; 13; 14; 15; 16]. Although it is clear that the cetacean thymus is a typical mammalian's [2; 4; 17] and that the T-cells depletion ('involution') occurs over time, the age or rate of progression at which such phenomena occurs is still undefined for any marine mammal species.

Based on the present study and previous information, we may conclude that cetaceans' thymus was similar to the observed in domestic mammals and that their thymic involution is age related. The data presented in this study may contribute to a better understanding of the physiological processes related to the immunity of Odontoceti and Mysticeti during their growth.

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References

[1] Cavagnolo RZ. The immunology of marine mammals. Developmental and Comparative Immunology. 1979; 3:245–257.

[2] Cowan, DF. Involution and Cystic Transformation of the Thymus in the Bottlenose Dolphin, *Tursiops truncatus*. Veterinary Pathology. 1994; 31:648-653.

[3] Simpson JG, Gardner MB. Comparative microscopic anatomy of selected marine mammals. In: Ridgway SH, editor. Mammals of the sea. Biology and Medicine. Springfield: Charles C. Thomas; 1972. p. 298-418.

[4] Romano TA, Felten SY, Olschowka JA, Felten DL. A microscopic investigation of the lymphoid organs of the beluga, *Delphinapterus leucas*. Journal of Morphology. 1993; 215(3):261-287.

[5] Wunschmann A, Seibert U, Frese K. Thymic cysts in Harbor Porpoises (*Phocoena phocoena*) from the German North Sea, Baltic Sea, and waters of Greenland. Veterinary Pathology. 1999; 36:391–392.

[6] Geraci JR, Lounsbury VJ. Marine Mammals Ashore: a Field Guide for Strandings. Baltimore: National Aquarium; 2005.

[7] Boehm, T. Thymus development and function. Current Opinion in Immunology. 2008; 20(2):178-184.

[8] Tizard, IR. Veterinary Immunology. Saint Louis: Saunders; 2012.

[9] Cunningham CP, Kimpton WG, Holder JE, Cahill RN. Thymic export in aged sheep: a continuous role for the thymus throughout pre- and postnatal life. European Journal of Immunology. 2001; 31(3):802-11.

[10] Eroschenko VP. Di Fiore's atlas of histology with functional correlations. Philadelphia: Lippincott Williams & Wilkins; 2008.

[11] Press C McL, Landsverk T. Immune System. In: Eurell JA, Frappier BL, editors. Dellmann's Textbook of Veterinary Histology. Ames: Blackwell Publishing; 2012. p. 134-152.

[12] Cowan, DF, Smith TL. Morphology of the lymphoid organs of the bottlenose dolphin, *Tursiops truncatus*. Journal of Anatomy. 1999; 194:505-517.

[13] Lubis I, Ladds PW, Reilly LR. Age associated morphological changes in the lymphoid system of tropical cattle. Research in Veterinary Science. 1982; 32:270-277.

[14] Suster S, Rosai J. Histology of the normal thymus. The American Journal of Surgical Pathology. 1990; 14(3):284-303.

[15] Newman AJ. Cysts of branchial arch origin in the thymus of the Beagle. The Journal of Small Animal Practice. 1971; 12(12):681-685.

[16] Slijper EJ. Whales. New York: Cornell University Press. 1979.

[17] Cave AJE. Note on *Tursiops* visceral histology. In: Pilleri G, editor. Investigations on Cetacea, 11th edition. Switzerland: `Der Bund'; 1980. p. 11-113.